THE RELEVANCE OF SINGLET OXYGEN IN THE ENVIRONMENTAL PHOTOCHEMICAL TRANSFORMATION OF SELECTED PHARMACEUTICALS

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Table of Contents

Summary .................................................................................................................................................. 1
Zusammenfassung ................................................................................................................................. 3

Chapter 1. Introduction ............................................................................................................................ 6
  1.1 Fundamentals of Aquatic Photochemistry for Pollutant Dynamics .............................................. 7
  1.2 Reversible (Proton-Coupled) Electron Transfer Reactions: Dissolved Organic Matter’s Role as a Sensitizer and a Quencher ................................................................................. 7
  1.3 Singlet Oxygen Reactivity with Organic Molecules, Amino Acids, and Peptides .................. 8
  1.4 Experimental Determination of Reactivity with Singlet Oxygen: Pros and Cons ..................... 9
  1.4 Scope of Discussion: Knowledge Gaps & Research Objectives ............................................. 10
  1.5 Thesis Organization ....................................................................................................................... 11
  1.6 References .................................................................................................................................... 13

Chapter 2. Environmental Photochemistry of Fenamate NSAIDs and their Radical Intermediates ... 16
  Abstract ................................................................................................................................................ 17
  2.1 Introduction .................................................................................................................................... 18
  2.2 Material and Methods .................................................................................................................... 19
    2.2.1 Simulated Sunlight Exposure ................................................................................................. 20
    2.2.2 Reactivity with Singlet Oxygen ............................................................................................. 20
    2.2.3 Reactivity with Triplet Sensitizer and Antioxidants .............................................................. 22
    2.2.4 Sample Analysis ..................................................................................................................... 22
    2.2.5 Transient Absorption Spectroscopy ...................................................................................... 22
  2.3 Results and Discussion .................................................................................................................... 23
    2.3.1 Photodegradation in Simulated Sunlight .............................................................................. 23
    2.3.2 Role of Reactive Oxygen Species ......................................................................................... 25
    2.3.3 Role of Triplet Sensitizing and Antioxidant Moieties in DOM ........................................ 26
    2.3.4 Radical Intermediates and Electron Transfer Properties .................................................... 28
  2.4 Implications ..................................................................................................................................... 31
  2.5 References ..................................................................................................................................... 32

Chapter S2. Supplementary Information: Environmental Photochemistry of Fenamate NSAIDs and their Radical Intermediates ................................................................. 35

Chapter 3. Non-Singlet Oxygen Kinetic Solvent Isotope Effects in Aquatic Photochemistry .......... 51
  Abstract ................................................................................................................................................ 52
  3.1 Introduction .................................................................................................................................... 53
  3.2 Experimental Section ..................................................................................................................... 55
    3.2.1 Materials and Solutions ......................................................................................................... 55
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Summary

Pharmaceuticals can contribute greatly to our daily lives, but after they are used for their intended purpose, it is important to understand what happens to them in the environment. To varying degrees, pharmaceuticals are excreted unaltered from humans and animals that are administered the drug. Pharmaceuticals can enter the environment when they are not completely removed during wastewater treatment, or given to animals whose excrements are then used as fertilizer. As pharmaceuticals are designed to have a biological effect, their presence in the natural environment can severely impact non-target organisms. To evaluate this risk, one needs to understand how long the pharmaceuticals remain in the environment. The main removal mechanisms for pharmaceuticals are adsorption to particles, biological, and chemical transformation. Herein, the effects of sunlight and with that, phototransformation reactions in surface water are given particular attention. For sunlit natural waters, phototransformation can occur directly by interaction with light or indirectly by the reaction with photochemically produced reactive intermediates, such as singlet oxygen.

The goals of this dissertation were to investigate the (photo)transformation pathways, with a particular focus on the reaction with singlet oxygen, for two classes of pharmaceuticals (i) anti-inflammatory drugs with a common diarylamine moiety and (ii) antimicrobial cyclic peptide drugs. The three main objectives of this work were to:

1. assess the environmental chemistry of fenamate nonsteroidal anti-inflammatory drugs and their radical intermediates,
2. quantify the non-singlet oxygen kinetic solvent isotope effects in aquatic photochemistry, and
3. determine the fate processes for the antimicrobial peptides daptomycin, bacitracin, and polymyxins in environmental systems.

To address the first objective, steady-state experiments were performed in simulated sunlight with and without dissolved organic matter (DOM), to evaluate the contribution of direct and indirect phototransformation reactions for five anti-inflammatory drugs. Direct photodegradation rates were markedly different for the structurally similar diarylamines based on variation in their light absorbance and subsequent reactivity (i.e. quantum yields). For indirect phototransformation, steady-state concentrations of major photochemically produced reactive oxygen species were quantified, and bimolecular reaction rate constants with singlet oxygen and hydroxyl radicals were calculated. Even though DOM acted as a photosensitizer and source for the reactive oxygen species, DOM also slowed down the phototransformation of diarylamines. Radical intermediates of diarylamines were visualized by sophisticated laser systems to demonstrate that their photodegradation was quenched by model antioxidants resembling moieties present in DOM. This quenching converts the radical intermediates back to the intact parent diarylamine and their observed phototransformation is slower.
A systematic comparison of methods to determine a compound's reactivity with singlet oxygen revealed that the method based on a kinetic solvent isotope effect needed further evaluation, which the second objective addressed herein. The kinetic solvent isotope effect was explored when changing the solvent from H₂O to D₂O, not only for the known reaction with singlet oxygen, but also involving effects by DOM itself. The lifetime of triplet excited compounds (photosensitizers) and their reaction kinetics with diarylamines did not show a solvent isotope effect. The quenching of photochemically-produced radical intermediates of diarylamines by a model antioxidant did, however, proceed slower in D₂O compared to H₂O. Slower quenching manifests itself in faster observed loss of the parent diarylamine in D₂O that will be observed as a kinetic solvent isotope effect. Without considering this additional solvent isotope effect, the reactivities with singlet oxygen would be overpredicted for all diarylamines tested by 5-100%.

The third objective addressed the environmental fate of peptide-based antimicrobial peptides. The phototransformation rates of the antimicrobial peptides were always enhanced in the presence of DOM. Singlet oxygen played a significant role, even for one peptide class, (polymyxins), that was not expected to react based on known singlet oxygen reaction pathways. In addition to phototransformation, all antimicrobial peptides showed moderate to high affinities to sorb to soil particles. These are the first empirical partitioning coefficients for the selected antimicrobial peptides and have orders of magnitude less variation than model-based octanol-water partitioning coefficients used in risk assessment for these compounds thus far. Besides abiotic processes, additional experiments demonstrate a biotransformation potential of these antimicrobial peptides by riverine biofilm communities.

This work provides an improved understanding of the environmental photochemistry of diarylamine pharmaceuticals and antimicrobial peptides. For both compound classes, the reaction with singlet oxygen played a central role. For diarylamines, a novel kinetic solvent isotope effect was identified that must be considered when evaluating the reactivity of a compound with singlet oxygen. For the reaction of the polymyxin antimicrobial peptides, unexpected reactivity with singlet oxygen offers the opportunity for future studies to explore a potential novel reaction mechanism.
Zusammenfassung


Die Ziele dieser Dissertation waren die (Photo-)Transformationspfade, insbesondere mit Singulett-Sauerstoff, für zwei Gruppen von Pharmazeutika zu untersuchen, namentlich (i) entzündungshemmende Schmerzmittel basierend auf einer Diarylamin struktur und (ii) antimikrobielle zyklische Peptide. Die drei Hauptziele der Dissertation waren:
(1) Bestimmung der Umweltphotochemie von diarylaminbasierten Entzündungshemmern und deren radikalen Zwischenprodukten,
(2) Quantifizieren des kinetischen Lösungsmittel-Isotopeneffektes durch die Reaktion mit Singulett-Sauerstoff und anderen photochemischen Reaktionen, und,
(3) Ermittlung von Verteilungs- und Transformationsprozessen für die antimikrobielle Peptide Daptomycin, Bacitracinen, und Polymyxinen in Umweltsystemen.

reaktiven Sauerstoffverbindungen fungiert, hatte das DOM auch eine hemmende Wirkung auf den Photoabbau der Diarylaminverbindungen. Radikal-basierte Zwischenprodukte der Diarylaminverbindungen wurden mittels hochentwickelter Lasersysteme beobachtet und deren Abbau wurde durch Antioxidationsmittel, die als Model für DOM-Strukturen dienen, unterdrückt. Diese Unterdrückung basiert darauf, dass die radikal-basierten Zwischenprodukte zur Ausgangssubstanz zurückreagieren und daher ein langsamerer Photoabbau dieser Arzneimittel beobachtet wurde.


Diese Arbeit liefert ein besseres Verständnis der Umwelphotochemie von diarylaminbasierten Arzneimitteln und antimikrobiellen Peptiden. Für beide Substanzklassen spielt die Reaktion mit Singulett-Sauerstoff eine entscheidende Rolle. Für die Diarylamine wurde ein neuer kinetische
Chapter 1. Introduction

Parts of this chapter have been published in:


1.1 Fundamentals of Aquatic Photochemistry for Pollutant Dynamics

To evaluate the relevance of singlet oxygen (\(^1\text{O}_2\)) in the environmental phototransformation of selected pharmaceuticals, one must also investigate the other key players in aquatic photochemistry.

For compounds with absorption spectra that overlap with the solar spectrum, direct photochemistry can contribute significantly to their photochemical transformation. During direct photochemical reactions, a molecule becomes electronically excited by absorbing light. From the excited state, the molecule can undergo a transformation reaction or be converted back to the parent compound by releasing energy to the solvent, by internal conversion, or emission of light (i.e., fluorescence). A molecule’s direct photochemical quantum yield (\(\Phi\)) describes how efficient the transformation process is, i.e., how many molecules are transformed in relation to how many photons of light are absorbed.

Indirect photochemical reactions are considered any reactions where light is absorbed by a molecule. These light absorbing molecules are called “(photo)sensitizers”. In their photochemically excited state, the sensitizers can react directly with the compound of interest by energy transfer or electron transfer, defined by their excited state triplet energy and redox potential. In addition, the excited sensitizer can react further to form additional reactive molecules that, in turn, react with the compound of interest.

Natural waters contain dissolved natural organic matter, including humic substances, that are formed from biotic and abiotic degradation of plant and animal materials, or synthesized by microorganisms.\(^1\) The subset of dissolved organic matter that is capable of absorbing light is called chromophoric dissolved organic matter or CDOM.

In experimental set-ups, model compounds are used as sensitizers for the purpose of evaluating photochemical processes. These model sensitizers are, discrete low-molecular weight chromophoric molecules with characterized triplet energies and redox potentials, and can be used to probe specific indirect photochemical reaction mechanisms. Conversely, CDOM, often from whole water samples or isolated humic substances, consists of mixtures of potentially thousands of sensitizer compounds. Hence, CDOM is used experimentally to get a more holistic understanding of the natural photochemical processes that are initiated by triplet excited CDOM (\(^3\text{CDOM}\)^\(^*\)). There are many photochemically produced intermediates (PPRIs) forming from \(^3\text{CDOM}\)^\(^*\) natural surface waters, including aqueous electrons, organoperoxy radicals, superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen.\(^2\) Besides the \(^3\text{CDOM}\)^\(^*\) itself, this dissertation especially highlights one major player: singlet oxygen (\(^1\text{O}_2\)).

1.2 Reversible (Proton-Coupled) Electron Transfer Reactions: Dissolved Organic Matter’s Role as a Sensitizer and a Quencher

Among all of the PPRIs, \(^3\text{CDOM}\)^\(^*\) is arguably one of the least understood due to the fact that it is not a single molecule, but is composed of a complex pool of many different chromophoric compounds
Chapter One: Introduction

with a range of excited state energies and redox potentials. A triplet excited state is produced when a ground state molecule absorbs a photon of light, is promoted to a highly energetic singlet excited state, then undergoes intersystem crossing, and is converted to the longer-lived excited triplet state. The $^{3}\text{CDOM}^*$ plays a central role in indirect photochemistry because it can not only react directly with a compound of interest, $^{3}\text{CDOM}^*$ is also the source of other PPRIs. $^{3}\text{CDOM}^*$ has a proclivity to oxidize aniline and phenol, with numerous examples of pollutant molecules documented in the literature. Beyond its well-known role as a photosensitizer, natural dissolved organic matter, including non-chromophoric molecules, can also be redox active in the electronic ground state. Phenolic moieties in organic matter have been demonstrated to act as an electron donor. Moreover, this antioxidant capacity of dissolved organic matter has been shown to inhibit the phototransformation of compounds that form radical intermediates.

1.3 Singlet Oxygen Reactivity with Organic Molecules, Amino Acids, and Peptides

The presence of $^{1}\text{O}_2$ is tied directly to the presence of $^{3}\text{CDOM}^*$, as it is the product of an energy transfer reaction between $^{3}\text{CDOM}^*$ and molecular oxygen ($^{3}\text{O}_2$). This energy transfer can occur for most triplet states because the energy required to excite molecular oxygen to $^{1}\text{O}_2$ is relatively low ($E_s = 94 \text{ kJ mol}^{-1}$). Common steady-state concentrations of $^{1}\text{O}_2$ in sunlit surface waters range from $10^{-12}$ to $10^{-14} \text{ M}$. Current knowledge about $^{1}\text{O}_2$ reactivity suggests that it is a rather selective oxidant, with the major reaction pathways being: $[2+2]$ and $[2+4]$ cycloaddition, ene reactions, phenol and sulfide oxidation. Several organic molecules known to undergo oxidation by $^{1}\text{O}_2$ include cyclic dienes, polycyclic aromatic compounds, heterocycles, as well as olefins containing allylic hydrogen atoms.

Several amino acids, freely dissolved in solution, are also known to be susceptible to varying degrees of photooxidation by $^{1}\text{O}_2$, namely cysteine (Cys), histidine (His), methionine (Met), tryptophan (Trp) and tyrosine (Tyr), from studies both in the context of environmental photochemistry of sunlit natural waters, and medical studies focusing on intracellular processes of reactive oxygen species in the context of oxidative stress. For combined amino acids, the peptide bonds have only a minor effect on $^{1}\text{O}_2$ lifetimes and are not a major site of reaction in biomolecules. Other studies relating $^{1}\text{O}_2$ reactivity of freely dissolved amino acids to amino acid residues within peptide and protein structures found that the local environment of the amino acid, from its primary protein structure, (i.e., amino acid sequence within a polypeptide chain), up to the tertiary protein structure, (i.e. overall three-dimensional structure, and accessibility of amino acid residues to the solvent) has a significant impact on its reactivity with $^{1}\text{O}_2$. 
1.4 Experimental Determination of Reactivity with Singlet Oxygen: Pros and Cons

Several experimental methods have been employed to determine the reactivity of $^1$O$_2$ with a compound of interest, all harbouring experimental advantages and potential disadvantages that can lead to over- or underprediction, as detailing in the following.

1.) **Photochemical Generation and Monitoring of $^1$O$_2$: by Laser Flash Photolysis**

In this experiment, $^1$O$_2$ is generated by a laser beam that photochemically excites a model triplet sensitizer, which reacts with molecular oxygen in solution. Singlet oxygen phosphorescence is monitored via a near-IR photo-multiplier tube and differences in the lifetime could be used to study the reaction with a compound of interest in solution. Until recently, these type of measurements had to be performed in D$_2$O because only the longer lifetime of $^1$O$_2$ in this medium made the detection possible.$^{33,34}$ State-of-the-art laser equipment allow this now to be measured directly in H$_2$O.$^{35}$ The singlet oxygen phosphorescence signal is fitted for exponential growth ($k_{\text{growth}}$) and decay ($k_{\text{decay}}$), and the lifetime of $^1$O$_2$ is determined as $1/k_{\text{decay}}$. The quenching effect of a compound of interest is assessed by determining $k_{\text{decay}}$ of $^1$O$_2$ in the presence of increasing concentrations of this compound of interest (Stern-Volmer plot). The slope of a linear regression of $k_{\text{decay}}$ values over quencher concentration yields the bimolecular reaction rate constant with $^1$O$_2$ ($k_{\text{tot}}$, M$^{-1}$s$^{-1}$). This rate constant is comprised of both physical and chemical quenching of $^1$O$_2$. An advantage of this technique is that it provides an upper limit for the reactivity of a compound with $^1$O$_2$. When considering the reactivity of a compound with $^1$O$_2$, however, one is mainly concerned with the chemical quenching, i.e., when $^1$O$_2$ reacts chemically to oxidize the compound of interest. Consequently, one overestimates the chemical reactivity, if the compound is able to physically quench $^1$O$_2$. Another drawback of this method is that it requires an elaborate laser flash photolysis set-up, and an ultrafast $^1$O$_2$ phosphorescence detector, which is not widely accessible to most researchers.

2.) **Photochemical Generation of $^1$O$_2$ by Dye-sensitized Steady-State Photolysis Experiments**

Singlet oxygen is generated by exciting a sensitizer that absorbs light in the visible range (>455 nm), i.e., a dye, which then reacts with molecular oxygen.$^{5,9,10,18,36,37}$ Common dyes employed in photochemistry for this purpose are Rose Bengal and methylene blue. Contrary to monitoring the transient $^1$O$_2$ directly in the laser set-up, this experiment is referred to as a steady-state experiment because only stable compounds are monitored in solution. Thus, degradation kinetics of the compound of interest are compared to those of furfuryl alcohol (FFA), a molecular probe for $^1$O$_2$. Furfuyl alcohol reacts predominantly with $^1$O$_2$ with a known bimolecular reaction rate constant.$^{38}$ An advantage of this method is that it requires relatively affordable and accessible equipment (light source and detector) and many compounds of interest do not absorb the visible light, minimizing direct photochemical transformation during the measurement. A drawback for this type of steady-state experiment is that it is impossible to generate $^1$O$_2$ without a triplet excited state. For compounds that
can also be oxidized by triplet excited states, additional experiments are necessary to differentiate the reaction mechanisms.

3.) **Photochemical Generation of $^{1}\text{O}_2$ Utilizing Kinetic Solvent Isotope Effect on the $^{1}\text{O}_2$ Lifetime**

In this method, $^{1}\text{O}_2$ is produced photochemically using a sensitizer and the reaction kinetics of the compound of interest are monitored in light and heavy water, i.e. $\text{H}_2\text{O}$ and $\text{D}_2\text{O}$, respectively.\(^5\)\(^9\)\(^10\)\(^18\)\(^36\)\(^37\) In $\text{D}_2\text{O}$, the steady-state concentrations of $^{1}\text{O}_2 ([^{1}\text{O}_2]_{ss})$ are higher because the main deactivation pathway of $^{1}\text{O}_2$ by transferring energy to the solvent, is much less efficient in $\text{D}_2\text{O}$. In $\text{H}_2\text{O}$, the overlap of the vibrational frequencies of O-H bonds matches the energy between ground state oxygen and the electronically excited $^{1}\text{O}_2$, which is not the case for $\text{D}_2\text{O}$. This phenomenon is referred to as a kinetic solvent isotope effect. Only if a compound of interest reacts with $^{1}\text{O}_2$, would one expect to observe faster degradation in $\text{D}_2\text{O}$. This method has been used in the past to determine bimolecular reaction rate constants, or as evidence that a compound of interest is generally susceptible to oxidation by $^{1}\text{O}_2$. An advantage of this method is that it requires a simple set-up present in most laboratories equipped to study environmental chemistry. A potential disadvantage of this method however is that one needs to ensure that changing the solvent to $\text{D}_2\text{O}$ does not alter concurrent reactions.

4.) **Thermal Generation of $^{1}\text{O}_2$ with Molybdate-Catalyzed Degradation of Hydrogen Peroxide**

Here, $^{1}\text{O}_2$ is generated chemically by the reaction of molybdate ($\text{MoO}_4^{2-}$) with hydrogen peroxide ($\text{H}_2\text{O}_2$).\(^5\)\(^39\)\(^40\) This method has the advantage that it is a way to produce $^{1}\text{O}_2$ without irradiation and without the generation of triplet excited states. This method, however, requires basic conditions (pH 10) and high $\text{H}_2\text{O}_2$ concentrations, which could be disadvantageous. One needs to ensure that base-catalyzed hydrolysis with the compound of interest or oxidation by hydrogen peroxide are minimal, or can be accounted for.

1.4 **Scope of Discussion: Knowledge Gaps & Research Objectives**

The overall goal of this dissertation was to study the pathways governing the environmental (photochemical) fate of two classes of pharmaceuticals.

One group consisted of five anthropogenic diarylamine-based anti-inflammatory drugs. These are low molecular weight compounds and classified as fenamates due to their shared fenamic acid moiety. Diclofenac, while technically not a fenamate due to a methylene ($\text{CH}_2$) separating the diphenylamine substructure from the acid moiety, was also included in this set because of its structural similarity to the fenamates, and the shared mode of action among these pharmaceuticals. Although photochemical studies had been performed for diclofenac\(^41\)\(^44\) and the fenamates,\(^45\)\(^46\) uncertainties regarding their indirect photochemical transformation pathways remained. These previous experiments concluded that the reaction of these drugs with PPRIs were not significant because adding PPRI quenchers did not slow down, but rather enhanced the observed reaction kinetics. No direct experimental evidence of reaction of these fenamates with PPRIs, beyond quenching experiments, were done prior to the
presented dissertation. In particular, the reaction of the fenamates with $^1$O$_2$ required further attention. The current method of choice for determining bimolecular reaction rate constants of compounds of interest with $^1$O$_2$, using the kinetic solvent isotope effect, had not been tested for its influences on other concurrent photochemical reactions. Evidence of a kinetic isotope effect on proton-coupled electron transfer reactions emphasized the need to evaluate this $^1$O$_2$ method for compounds like fenamates that also undergo redox reactions from CDOM triplet excited states and DOM antioxidant moieties.\textsuperscript{6, 47}

The second group of pharmaceuticals under investigation consisted of three high molecular weight antimicrobial peptides of natural origin. These antimicrobial peptides are applied not only as a therapeutic, but also as growth-promoters in livestock\textsuperscript{48, 49} The interest in antimicrobial peptides increased strongly in the past decade because they are considered a last-resort to multi-drug resistant bacteria.\textsuperscript{50, 51} While recent studies assessed the reactivity of bacitracin A with singlet oxygen\textsuperscript{52}, and affinity to environmental soil matrices\textsuperscript{53, 54}, no experimental data exist regarding any environmental fate processes for daptomycin and the polymyxins. In addition to phototransformation reactions, the sorption behaviour and biotransformation potential for bacitracins, daptomycin, and polymyxins were investigated in this dissertation.

The specific objectives of this dissertation were to:

1: Assess the environmental photochemistry of fenamate nonsteroidal anti-inflammatory drugs and their radical intermediates (Chapter 2)

2: Quantify the non-singlet oxygen kinetic solvent isotope effects in aquatic photochemistry (Chapter 3)

3: Determine the fate processes of the antimicrobial peptides daptomycin, bacitracin, and polymyxins in environmental systems (Chapter 4)

1.5 Thesis Organization

Chapter Two: This chapter investigated the photochemical transformation of a group of nonsteroidal anti-inflammatory drugs (i.e. diclofenac, and the fenamates - flufenamic acid, meclofenamic acid, mefenamic acid and tolfenamic acid). Steady-state experiments were performed with simulated sunlight, both in the presence and absence of natural organic matter. Indirect photochemical pathways were also targeted with experiments performed in UVA light with model sensitizers and probe molecules, to determine reaction rate constants with environmentally relevant photochemically produced reactive intermediates ($^*$OH, $^3$sens*, and $^1$O$_2$). Transient absorption spectroscopy allowed for visualization of the quenching effect caused by the fenamates on the lifetime of triplet excited sensitizer molecules (perinaphthenone and lumichrome). For the first time, short-lived radical
intermediates of the fenamates were observed following sensitization with excited triplet sensitizers. The quenching rates of the radical intermediates by a model antioxidant, ascorbic acid, were quantified.

Chapter Three: This chapter provided a systematic assessment of deuterated solvent isotope effects on reaction kinetics of photochemical transformation reactions, using the fenamate-based NSAIDs as model compounds. The singlet oxygen lifetime was measured using singlet oxygen phosphorescence for 12 compositions of H$_2$O:D$_2$O to establish a relationship between solvent composition and kinetic decay rates constants. $^1$H-NMR, $^{13}$C($^1$H)NMR, and high resolution mass spectrometry were used to confirm H/D exchange and incorporation of deuterium with model sensitizers representative of those found in dissolved organic matter. Transient absorption spectroscopy was used to investigate deuterated solvent effects on the lifetime of triplet excited model sensitizers, as well as their reactivity with NSAIDs. Finally, the quenching rate constants of antioxidants with NSAID radical intermediates were compared in H$_2$O and in D$_2$O. Steady-state experiments were also performed in H$_2$O and D$_2$O under the following conditions: without sensitizers, with DOM as a sensitizer, with model sensitizers perinaphthenone and model antioxidant caffeic acid, under argon sparged conditions, and in the presence of superoxide dismutase.

Chapter Four: This chapter investigated the environmental behaviour of antimicrobial peptides and required initial analytical method development including liquid chromatography coupled to in-needle derivatization and detection with fluorescence, as well as high-resolution tandem mass spectrometry. Sorption experiments to determine soil-partitioning coefficients (K$_d$) were adapted from the OECD Guideline 106: Adsorption – Desorption using a Batch Equilibrium Method, and were performed for two standardized European Soils (sandy and clayey). Steady-state photochemical experiments were performed in simulated sunlight with and without dissolved organic matter, to determine direct and indirect phototransformation pathways. The bimolecular reaction rate constants of antimicrobial peptides with O$_2$ was determined by exciting the model sensitizer perinaphthenone in UVA light and employing the kinetic solvent isotope effect method. Finally experiments adapted from the OECD 309: Aerobic Mineralization in Surface Water – Simulation Biodegradation Test were performed using concentrated riverine biofilms to determine the biotransformation potential of the selected antimicrobial peptides.

Chapter Five: This chapter provides a general conclusion and an outlook for further extensions of the presented work.
Chapter One: Introduction

1.6 References


Chapter 2. Environmental Photochemistry of Fenamate NSAIDs and their Radical Intermediates

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Abstract
Fenamates are a class of non-steroidal anti-inflammatory drugs (NSAIDs) that are not fully removed during wastewater treatment and can be released to surface waters. Here, near-surface photochemical half-lives were evaluated to range from minutes to hours of four fenamates and the closely related diclofenac. While quantum yields for direct photochemical reactions at the water surface vary widely from 0.071 for diclofenac to <0.001 for mefenamic acid, all fenamates showed significant reactivity towards singlet oxygen and hydroxyl radical with bimolecular reaction rate constants of $1.3-2.8 \times 10^7$ M$^{-1}$s$^{-1}$ and $1.1-2.7 \times 10^{10}$ M$^{-1}$s$^{-1}$, respectively. Photodecay rates increased in the presence of dissolved organic matter (DOM) for diclofenac (+19%), tolfenamic acid (+9%), and mefenamic acid (+95%), but decreased for flufenamic acid (-2%) and meclofenamic acid (-14%) after accounting for light screening effects. Fast reaction rate constants of all NSAIDs with model triplet sensitizers were quantified by laser flash photolysis. Here, the direct observation of diphenylamine radical intermediates by transient absorption spectroscopy demonstrates one-electron oxidation of all fenamates. Quenching of these radical intermediates by ascorbic acid, a model antioxidant, was observed. These observations suggest that the balance of oxidation by photoexcited triplet DOM and quenching of the formed radical intermediates by DOM constituents determines whether net sensitization or net quenching by DOM is observed in the photochemical degradation of fenamates.

Keywords: pharmaceuticals, transformation, antioxidant, diclofenac, diphenylamine
Chapter Two: Environmental Chemistry of Fenamate NSAIDs and their Radical Intermediates

2.1 Introduction

Fenamates are nonsteroidal anti-inflammatory drugs (NSAIDs) that contain fenamic acid (N-phenylanthranilic acid) as a core structural unit (Figure 1). Members of this family include mefenamic acid, flufenamic acid, meclofenamic acid, and tolfenamic acid. Diclofenac is closely related, having a methylene (CH$_2$) separating the diphenylamine substructure from the acid moiety. The therapeutic effect of pain relief is achieved by inhibiting cyclooxygenase enzymes, which facilitate the oxidation of arachidonic acid and initiate the downstream inflammation response in mammals, including humans.$^{55-58}$ Fenamate-based NSAIDs are excreted by humans and animals$^{59-62}$, are not always completely removed during wastewater treatment, and thus, high effluent concentrations can contribute to entry of these drugs into surface waters.$^{63-66}$

![Figure 1. Structures of fenamic acid and the fenamate NSAIDs mefenamic acid - 1, tolfenamic acid - 2, meclofenamic acid - 3, and flufenamic acid - 4, and diclofenac - 5.](image)

Mefenamic acid, marketed worldwide as Ponstel or Ponstan, has been detected in wastewater effluents at concentrations up to 1.4 $\mu$g L$^{-1}$ in Switzerland$^{67}$ and up to 0.14 $\mu$g L$^{-1}$ in Chinese surface waters.$^{68}$ Flufenamic acid (Flufen, Opyrin) a trifluoromethylated fenamate, is only sold in a limited number of countries, but was also detected in Spanish wastewater influent and effluent.$^{69}$ Other fenamate drugs, meclofenamic acid (Meclomen, Eucome) and tolfenamic acid (Clotam, Tolfedine) contain chloro and methyl groups, are primarily used in veterinary medicine, and are sold mostly in Asia and limited parts of Europe. So far, insufficient information is available about their concentration in surface waters because these fenamates have rarely been included in environmental screening studies. Few studies show that their concentrations were below the limit of detection (0.050 $\mu$g L$^{-1}$)$^{70,71}$, however, tolfenamic acid had been detected at up to 1.6 $\mu$g L$^{-1}$ in Brazilian wastewater treatment plant effluents.$^{72}$ Diclofenac, which is sold under various trade names such as Arthrotec, Cataflam, and Voltaren, is one of the most abundantly sold NSAIDs worldwide$^{73}$ and has been implicated in the mass killing of Asian vultures.$^{74}$ Consequently, diclofenac has been more extensively studied and has been detected in microgram per liter concentrations in various wastewater treatment plant influents and effluents$^{42, 67, 70, 71, 75-79}$, as well as in surface waters$^{70, 72, 80}$, and studies demonstrate no significant biodegradation.$^{81,82}$ While diclofenac is routinely monitored and wastewater treatment aims to limit its...
Chapter Two: Environmental Chemistry of Fenamate NSAIDs and their Radical Intermediates

discharge into surface waters, less attention has been given to fenamates. These pharmaceuticals exhibit the same mode of action (NSAIDs, inhibiting cyclooxygenases) and the co-occurrence of these drugs may lead to synergistic effects in the environment.

All fenamates have pK\textsubscript{a} values ranging from 3.7 to 4.3\textsuperscript{61, 83-86}, making them ionic at neutral pH. The estimated octanol-water partitioning coefficients for the ionic species, logD\textsubscript{ow}, range from 2 to 3 (Table S1 in the supplementary information, SI), and was experimentally determined as 0.68 for diclofenac\textsuperscript{87} and as 1.6 for mefenamic acid.\textsuperscript{88} These parameters suggest that sorption and sedimentation are not the most important pathways for removal of such compounds from the aqueous phase.

Once in surface waters, photochemical transformation processes may be some of the most relevant removal pathways of fenamates. Several previous studies focused on the direct photochemical transformation of diclofenac and estimated an environmental half-life in sunlight between 30 minutes to 1 hour in the top layer of a water column\textsuperscript{42, 44}, which is affected in natural water due to light screening by dissolved organic matter (DOM).\textsuperscript{76} The photochemical half-lives of fenamates have only been partially investigated, e.g., for flufenamic acid (1 hour, artificial light: 300-450 nm)\textsuperscript{46} and for mefenamic acid (33 hours, noon sunlight, 45° N latitude).\textsuperscript{45} So far, the indirect photochemical pathways, including transformation by reactive oxygen species or interactions with DOM for have not been studied in detail for these fenamates.

This study presents a detailed investigation of the environmental photochemical transformation kinetics and mechanisms of the fenamates and diclofenac by comparing direct and indirect photochemical degradation processes. While direct photodegradation dominates for some of the compounds at the water surface, the results demonstrate that reactions with reactive oxygen species and photochemically excited DOM are significant, especially deeper in the water column. In particular, the interactions with DOM as a natural photochemical sensitizer and antioxidant towards radical intermediates were directly observed and quantified by transient absorption spectroscopy.

2.2 Material and Methods

Materials. Experiments were carried out buffer from potassium phosphate dibasic (Sigma-Aldrich, ≥98%) and potassium dihydrogen phosphate (Fluka, ≥99.5%). Aqueous solutions were prepared with ultrapure water (>18 MΩ cm, Barnstead Nanopure Diamond system). The following reagents were all purchased from Sigma-Aldrich and used as received: acetonitrile (HPLC grade), methanol (HPLC grade), 2-hydroxyterephthalic acid (97%), L(+)-ascorbic acid sodium salt (≥99.5%), caffeic acid (≥98%), diclofenac sodium salt (≥98.5), lumichrome, meclofenamic acid sodium salt, perinaphthenone (97%), pyridine (Chromosolv ≥99.9%), Rose Bengal (95%), sodium acetate trihydrate (≥99.0%), sodium benzoate (BioUltra ≥99.5%), sodium nitrite (≥ 99%) and tolfenamic acid. Flufenamic acid (97%) was purchased from Acros Organics. 4-nitroanisole (Sigma-Aldrich, 97%) was recrystallized.
before use. The dipotassium terephthalate salt (K₂TPA) was prepared from terephthalic acid (Sigma-Aldrich, 98%) as described elsewhere. Acetic acid (≥99.8%), hydrogen peroxide (Trace Select ≥ 30%, no stabilizers), mefenamic acid (≥98%), and sodium azide (≥99.0%) were obtained from Fluka. Sodium molybdate dihydrate (≥99.5%) was purchased from Merck. Furfuryl alcohol (Merck, ≥ 98%) was distilled prior to use and kept under argon to prevent oxidation. Deuterium oxide (99.8 atom% D) was purchased from Armar Isotopes. Pony Lake Fulvic Acid (1R109F) and Suwannee River Fulvic Acid (2S101F) were purchased from the International Humic Substance Society (IHSS).

Methods. All light exposure tests were performed with 5 µM test compounds (1-5) in phosphate buffer (5 µM, pH 7.5) and dark controls were included, unless stated otherwise.

2.2.1 Simulated Sunlight Exposure. Compounds 1-5 were individually exposed to simulated sunlight (Heraeus model Suntest CPS+) in open quartz test tubes, positioned at a 20° angle from the horizontal plane, 30 cm below the light source, and submerged in a temperature-controlled water bath (27°C ± 1°C). Furfuryl alcohol (FFA, 40 µM) was used for quantification of singlet oxygen. Additional samples were prepared containing the humic substance isolate Pony Lake Fulvic Acid (PLFA, 10 mg carbon L⁻¹). Aliquots were taken in triplicates and analyzed for the test compound and FFA as described below.

To calculate the quantum yield of direct photochemical reactions, the chemical actinometer system PNA-PYR (10 µM p-nitroanisole, 0.5 mM pyridine) were irradiated with simulated sunlight in ultrapure water in identical test tubes alongside the test compounds. The quantum yields for the test compounds, \( \phi_{\text{test comp.}} \), are expressed as:

\[
\phi_{\text{test comp.}} = \phi_{\text{act.}} \cdot \left( \frac{k_{\text{test comp.}}}{k_{\text{act.}}} \right) \cdot \frac{\sum_k \varepsilon_k L_k \lambda_{\text{rel.}}}{\sum_k k_{\text{act.}} L_k \varepsilon_k \lambda_{\text{rel.}}} \tag{1}
\]

with the observed degradation rate constants \( k \) (s⁻¹), the quantum yield of the actinometer \( \phi_{\text{act.}} \) being 0.29[PYR] + 0.00029²⁹, the wavelength dependent molar absorbivities \( \varepsilon \), and relative light irradiance of the simulated sunlight.²⁹

2.2.2 Reactivity with Singlet Oxygen. To determine the bimolecular reaction rate constants of compounds 1-5 with singlet oxygen, \( ^1\text{O}_2 \), four different methods have been evaluated. The methods include photochemically sensitized experiments with Rose Bengal, time-resolved \( ^1\text{O}_2 \) phosphorescence quenching, non-photochemical generation of \( ^1\text{O}_2 \) by hydrogen peroxide and molybdate, and finally the evaluation of the kinetic solvent isotope effect (KSIE) in D₂O.⁵,⁹² The former three methods may produce artifacts due to high reactivity of the test compounds with triplet excited dyes, contribution of physical quenching, and instability at high solution pH required, respectively. Thus, the KSIE method was chosen to determine the reaction rate constants. Details about these methods can be found in the ESI (Text S1 and Figure S1). The KSIE method depends on an increase
in $^{1}$O$_2$ lifetime in D$_2$O that is reflected by a higher $^{1}$O$_2$ steady-state concentration, $[^{1}$O$_2]_{ss}$. Faster degradation of compounds 1-5 in D$_2$O can be quantitatively attributed to the reaction with $^{1}$O$_2$. For the KSIE tests, samples were prepared with 5 µM compounds 1-5, 0.77 µM perinaphthenone, and 40 µM FFA as the $^{1}$O$_2$ probe in either H$_2$O or approximately 90% D$_2$O at pH 7.5 (phosphate buffer, 5 mM, Text S2 and Table S2). Samples were irradiated in open borosilicate test tubes with enhanced UVA light (2 bulbs, centered at 365 nm) on a turn table in a Rayonet photoreactor (Southern New England Ultraviolet Company, Branford, USA) with a polymer heat/bandpass filter situated between the lamps and the samples to remove light below 320 nm (269 LEE Heat Shield, Lee Filters, Hampshire, UK), in addition to long wavelengths (> 400 nm). The bimolecular reaction rate constant with $^{1}$O$_2$, $k_{rxn,1O2}$, was estimated as:

$$k_{rxn,1O2} = \frac{k_{D2O}-k_{H2O}}{[^{1}O_2]_{ss,D2O}-[^{1}O_2]_{ss,H2O}}$$  \hspace{1cm} (2)

with the observed decay rate constants in D$_2$O ($k_{D2O}$) and in H$_2$O ($k_{H2O}$) and the respective steady-state concentrations of $^{1}$O$_2$, $[^{1}$O$_2]_{ss}$ calculated from the observed decay rates of FFA at 23ºC with a known reaction rate constant of 1.03 ± 0.01 x 10$^8$ M$^{-1}$s$^{-1}$ (specific for this temperature) as detailed in Text S3.$^5$

2.2.3 Reactivity with Hydroxyl Radicals. The bimolecular reaction rate constants with hydroxyl radical, $^{•}$OH, were determined using benzoic acid as a reference compound. Samples were prepared with 10 µM sodium benzoate, and 1 mM H$_2$O$_2$ and were irradiated in a Rayonet photoreactor with enhanced UVA light (emission centered at 365 nm, 8 bulbs). Sodium nitrite (6.5 µM) was used as a $^{•}$OH source for meclofenamic acid because not enough $^{•}$OH were produced from H$_2$O$_2$ to distinguish decay due to $^{•}$OH vs. direct photochemical decay. Control samples without H$_2$O$_2$ (or nitrite) were also tested. Competition plots were generated by plotting the normalized decay of test compounds, ln(C/C$_0$) against that of benzoic acid and the slope, S, was determined by linear regression.

The bimolecular reaction rate constant of test compounds was assessed as:

$$k_{rxn,•OH} = S \cdot k_{rxn(BZA)}$$ \hspace{1cm} (3)

with $k_{rxn(BZA)}$ being the bimolecular reaction rate constant of benzoic acid (5.9 ± 0.1 x 10$^9$ M$^{-1}$s$^{-1}$).$^{33}$ The steady-state concentration of $^{•}$OH, $[^{•}$OH]$_{ss}$, produced by DOM (10 mg L$^{-1}$ PLFA) in the solar simulator was quantified using terephthalic acid (TPA, 10 µM) as the $^{•}$OH probe by monitoring the formation of hydroxylated product 2-hydroxyterephthalic acid (hTPA).
2.2.3 Reactivity with Triplet Sensitizer and Antioxidants. To determine the reactivity of compounds 1-5 with photochemically excited triplet sensitizers, perinaphthenone was used as a model sensitizer. Samples contained 0.77 µM of perinaphthenone and were irradiated in open borosilicate test tubes with enhanced UVA light (2 bulbs, with heat/bandpass filter) on a turn table in a Rayonet photoreactor. Additional experiments were conducted with identical samples, but sealed and sparged with argon for 15 minutes prior to irradiation to remove O\(_2\), an effective triplet quencher. Control samples without perinaphthenone were also included. Additional tests were performed in the presence of a model antioxidant, 10 µM caffeic acid (3,4-dihydroxycinnamic acid). Caffeic acid represents a plant-derived diphenoxy-based reducing agent with relatively low absorbance of UVA light, which minimizes the direct photodecay in these tests.

2.2.4 Sample Analysis. Samples were analyzed for compounds 1-5 by Ultra Performance Liquid Chromatography (UPLC) on a C18 column (Waters Acquity, BEH 130 C18, 1.7 µm, 2.1×150 mm), injection volume of 5 µL, 0.20 ml min\(^{-1}\) flow rate with an isocratic method of eluent (A) 0.1% formic acid with 10% acetonitrile and (B) 100% acetonitrile at a ratio of 20:80 (A:B) and detection by absorbance at 288 nm. Benzoic acid was analyzed with an eluent ratio of 70:30 (A:B) by absorbance detection at 245 nm. 2-hydroxyterephthalic acid was analyzed with an eluent composition of (A) 0.1% formic acid with 10% methanol (MeOH) and (B) 100% MeOH at 70:30 (A:B) and detection by fluorescence (excitation: 250 nm, emission: 410 nm). Furfuryl alcohol and p-nitroanisole were analyzed on a C18 column (Agilent Eclipse - XDB C18, 5 µm, 4.6×150 mm) at 1.0 ml min\(^{-1}\) flow rate with an eluent composition of (A) sodium acetate buffer (pH 5.9, 15.6 mM) and (B) 100% acetonitrile isocratically at a ratio of 90:10 and 40:60 (A:B), respectively and were detected by absorbance at 219 nm and 316 nm, respectively. All first-order degradation rate constants, \(k_{\text{obs}}\) (s\(^{-1}\)), were assessed as the slope of a linear regression of natural log-transformed normalized concentration, ln\((C/C_0)\), versus irradiation time.

2.2.5 Transient Absorption Spectroscopy. To further elucidate the reaction mechanisms of compounds 1-5 with triplet excited states, laser flash photolysis was used to (a) determine reaction rate constants with triplet sensitizer, (b) evaluate the formation of radical intermediates, and (c) quantify the reactivity of these radical intermediates with antioxidants. Perinaphthenone (PN) and lumichrome were chosen as model sensitizers and ascorbic acid as a model antioxidant. Perinaphthenone was selected as a conservative representative model sensitizer for CDOM due to its relatively low triplet energy (\(E_T = 164\) kJ mol\(^{-1}\)) and triplet state one-electron reduction potential (\(E^{\text{st}} = 1.03\) 3S*/S- V, SHE).\(^4\) Lumichrome was selected to generate fenamate radical intermediates in the laser system due to its higher reduction potential (\(E^{\text{st}} = 1.91\) 3S*/S- V, SHE).\(^4\) Both sensitizers were also suitable for laser experiments because their triplet signals did not overlap with the transient signals from the fenamates. Ascorbic acid was selected as a model antioxidant because of its high
Trolox equivalent antioxidant capacity (1.03 TEAC<sub>ABTS</sub>*<sup>95</sup>) and also because its absorption spectrum did not overlap with the excitation wavelengths used for the laser experiments.

Transient absorption spectroscopy was carried out using a pump-probe system (EOS, Ultrafast Systems, Sarasota, USA). Pump pulses were produced by a regeneratively amplified Ti:sapphire laser, (output of 3.5 W at 795 nm, 1 kHz Solstice, Newport Spectra-Physics, Irvine, USA), which were converted to the desired excitation wavelength of 365 nm using a TOPAS Optical Parametric Amplifier (Light Conversion, Vilnius, Lithuania). Samples contained 100 µM perinaphthenone and increasing concentrations of compounds 1-5 (100-1000 µM) in 50% acetonitrile continuously sparged with synthetic air. The time-dependent change in absorbance (ΔA) for the triplet-excited state feature (^3PN*, centered at 490 nm) was monitored. Transient absorbance traces were fit to exponential decay functions for lifetime estimates, τ (= 1/k<sub>obs</sub>) (OriginPro 9.0, OriginLab Corp. Northampton, MA). The bimolecular reaction rate constants, k^3PN*, of test compounds with the triplet sensitizer were obtained from the slope of linear regression of measured triplet decay rate constants, k<sub>obs</sub>(^3PN*), versus concentration of compounds 1-5 (Figure S2-5). The quenching rate constant of ^3PN* by the antioxidant, caffeic acid was also assessed in this manner (Figure S6).

To evaluate the formation of radical intermediates of the test compounds upon reaction with triplet sensitizer, further experiments were performed with lumichrome (100 µM, excitation at 370 nm), 400 µM compounds 1-5, in phosphate buffer at pH 6.0 with 50% acetonitrile, sparged continuously with argon. Transient absorbance spectra were evaluated for radical intermediates of compounds 1-5 expected to occur around 670 nm as previously observed for diphenylamine. ^96

Lastly, to evaluate the reactivity of the radical intermediates with antioxidants, the change of τ of radical intermediates was measured in the presence of increasing concentrations of ascorbic acid (100-1000 µM). The bimolecular reaction rate constant was assessed as the slope of the linear regression of the measured radical decay rate constants plotted versus concentration of ascorbic acid (Figure S7-S9).

2.3 Results and Discussion

2.3.1 Photodegradation in Simulated Sunlight. The photochemical half-lives in surface waters of the pharmaceuticals were evaluated by exposing aqueous solutions to simulated sunlight in the absence and presence of DOM (PLFA, 10 mg<sub>C</sub> L<sup>-1</sup>). Compounds 1-5 were irradiated individually. Data in Figure 2 show that the pseudo-first-order decay rates in the presence of DOM were substantial for compounds 1-5, but also varied significantly. In the presence of DOM, diclofenac was degraded the fastest (t<sub>1/2</sub> = 19 min), followed by flufenamic acid, meclofenamic acid, tolfenamic acid, and mefenamic acid (t<sub>1/2</sub> = 9.4 hours). The same order was observed in the absence of DOM (Figure S10).
Quantum yields for direct photochemical transformation ranged from < 0.001 for mefenamic acid to 0.071 for diclofenac (Table 1). Flufenamic acid has a slightly lower quantum yield than meclofenamic acid, but its higher molar absorptivity across the solar spectrum results in an overall faster direct photochemical decay (Figure S11).

Not only the overall rates, but also the contribution of direct and indirect photochemical processes varied significantly among compounds 1-5. The direct photochemical degradation was compared to the overall degradation in the presence of DOM after accounting for light screening effects (Text S5 and Table S3). The decay rate constant increased for tolfenamic acid, diclofenac and mefenamic acid in the presence of DOM, with 9%, 20%, and 95% of the overall photodegradation being attributed to indirect photochemical processes, respectively. The relative indirect contribution for mefenamic acid is so high due to its negligible direct photochemical decay. DOM acted as a net sensitizer towards these compounds. The observed degradation rates of flufenamic acid and meclofenamic acid decreased in the presence of DOM by 2% and 14% respectively, even after accounting for light screening effects. Thus, DOM acted as a net quencher towards these two fenamates.

Data in Figure 2B show modelled contributions of indirect photochemical processes in a water column (model description in Text S6, Figure S11-S12). The sunlight intensity decreases with depth because chromophoric components (e.g., DOM) absorb light and particularly shorter wavelengths in the UV
range do not penetrate deep into the water column. Thus, all photochemical decay processes rapidly decrease down the water column where less light is available. Even though direct photochemical reactions dominated for compounds 2-5 at the water surface (top 1 cm), the relative contribution of indirect photochemical processes becomes competitive below the top 10-40 cm (Figure 2B).

First, indirect photochemical processes including reactions with singlet oxygen, hydroxyl radicals, and triplet excited sensitizers were quantified to evaluate the net sensitizing effect of DOM. Then the role of model antioxidants towards the photochemistry of fenamates and diclofenac was investigated to demonstrate the underlying mechanism of net quenching effects of DOM.

Table 1. Net effect of DOM, quantum yields for direct photochemical reactions, reaction rate constants with singlet oxygen $k(^1\text{O}_2)$, hydroxyl radical $k(\cdot \text{OH})$, model triplet sensitizer (perinaphthenone, $k(3\text{PN}^*)$) for test compounds, and quenching rate constants of the radical intermediates of the test compounds with model antioxidant (ascorbic acid, $k(\text{AA})$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect of DOM</th>
<th>Quantum yield</th>
<th>Reaction Rate Constants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta k_{\text{obs}}$ (%)</td>
<td></td>
<td>$k(\cdot \text{O}_2)$ $\pm$ std. dev. $\times 10^n$ (M$^{-1}$ s$^{-1}$)</td>
</tr>
<tr>
<td>(1) Mefenamic acid</td>
<td>+ 95 ± 3</td>
<td>&lt; 0.001</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2) Tolfenamic acid</td>
<td>+ 9 ± 3</td>
<td>0.001</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) Meclofenamic acid</td>
<td>- 14 ± 2</td>
<td>0.010</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4) Flufenamic acid</td>
<td>- 2 ± 1</td>
<td>0.006</td>
<td>1.3 ± 0.2</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5) Diclofenac (2-phenylacetic)</td>
<td>+ 19 ± 2</td>
<td>0.071</td>
<td>n.d.$a$</td>
</tr>
</tbody>
</table>

*a*determined by kinetic solvent isotope effect (Figure S13); $^b*$no significant rate constant detected with any of the applied methods (Figure S1 and S13); $^c$compare to $2.10 \times 10^{10}$ M$^{-1}$ s$^{-1}$ by Aruoma et al. $^d$compare to $1.30 \times 10^{10}$ M$^{-1}$ s$^{-1}$ by Aruoma et al. $^e$compare to $7.50 \times 10^{9}$ M$^{-1}$ s$^{-1}$ by Huber et al. $f$no reaction rate constant was calculated because the radical intermediate of diclofenac was not detected under these conditions.

2.3.2 Role of Reactive Oxygen Species. Reactions with singlet oxygen ($^1\text{O}_2$) and hydroxyl radical ($\cdot \text{OH}$) can significantly contribute to the fate of some pollutants in surface waters. Thus far, the reaction
rate constants of $^1\text{O}_2$ with compounds 1-5 had not been determined and only some rate constants existed for $^1\text{OH}$.

The bimolecular reaction rate constants of compounds 1-5 with $^1\text{O}_2$ were obtained by rate comparison in H$_2$O versus D$_2$O (KSIE) and range from 1.3 to $2.8 \times 10^7$ M$^{-1}$s$^{-1}$ (Table 1). All fenamates react significantly with $^1\text{O}_2$ when compared to other pharmaceuticals for which reaction with $^1\text{O}_2$ was identified as a major decay process, for example ranitidine ($1.6 \times 10^7$ M$^{-1}$s$^{-1}$, pH 6.4)$^{23}$, cimetidine ($9.2 \times 10^7$ M$^{-1}$s$^{-1}$, pH 6.9)$^{23}$ or sulfathiazole ($5.5 \times 10^7$ M$^{-1}$s$^{-1}$)$^{100}$. No significant KSIE was observed for diclofenac and also in the $^1\text{O}_2$ phosphorescence experiments, increasing concentrations of diclofenac did not show measurable quenching of $^1\text{O}_2$.

The measured reaction rate constants with $^1\text{O}_2$ ($k_{\text{rxn,1O}_2}$) allow estimation of the relative contribution via reaction with $^1\text{O}_2$ to the overall photodegradation (Table S4). Therefore, $k_{\text{rxn,1O}_2}$ was multiplied by the steady-state concentrations of $^1\text{O}_2$ ranging from 2.9-3.3 $\times 10^{-13}$ M under simulated sunlight conditions (Figure 2A). The reaction pathway with $^1\text{O}_2$ was most important for mefenamic acid, with 24% of its total observed degradation in the presence of DOM. Tolfenamic, meclofenamic, and flufenamic acid had a lower contribution of reaction with $^1\text{O}_2$ of 10%, 4% and 1% of the total degradation, respectively.

The bimolecular reaction rate constants of compounds 1-5 with $^1\text{OH}$, $k_{\text{rxn,OH}}$, was obtained by competition experiments with benzoic acid and photochemical $^1\text{OH}$ generation (Figure S14). The reaction with $^1\text{OH}$ is rather unspecific and occurs at nearly diffusion-controlled rates for compounds 1-5 ranging from 1.0 to $2.7 \times 10^{10}$ M$^{-1}$s$^{-1}$. Analogous to the pathway with $^1\text{O}_2$, the contribution to the overall photodegradation via reaction with $^1\text{OH}$ under simulated sunlight conditions (Figure 2A) was determined by multiplying $k_{\text{rxn,OH}}$ with the steady-state concentration of $^1\text{OH}$. Despite the high reactivity with $^1\text{OH}$, the overall contributions for compounds 1-5 range only from 0.1-1.3% (Table S4) because of the low steady-state concentration of $^1\text{OH}$ of 2.4 $\times 10^{-17}$ M.

### 2.3.3 Role of Triplet Sensitizing and Antioxidant Moieties in DOM
Dissolved organic matter is redox active and can act as both a sensitizer and an antioxidant. Consequently, DOM can decrease or increase photochemical half-lives of organic molecules. Here, mefenamic acid, tolfenamic acid, and diclofenac underwent enhanced photochemical degradation in the presence of DOM with contribution of 95%, 9% and 20% to the overall decay rate constant, $k_{\text{obs}}$, respectively (Table 1). A summary of the contribution of reaction with $^1\text{O}_2$ and $^1\text{OH}$ to the overall indirect photodegradation can be found in Table S4. For tolfenamic acid, the 9% enhancement of photodegradation is seemingly explained by the reaction with $^1\text{O}_2$ (approx. 10%). For mefenamic acid and diclofenac however, a remaining 71% and 20% of the enhanced degradation, respectively, cannot be explained by the presence of reactive
oxygen species alone (i.e., $^1$O$_2$ and $^*$OH) and must come from additional reaction pathways. In contrast, the presence of DOM reduced the photochemical half-lives of meclofenamic acid and flufenamic acid by 14% and 2%, respectively.

The reaction mechanisms behind the dual roles of DOM as sensitizer and quenchers were investigated further by employing model triplet sensitizers and model antioxidants. Data in Figure 3A show the pseudo-first order decay curves for diclofenac during irradiation with UVA light, minimizing the influence of direct photochemical processes. Photodegradation was enhanced in the presence of the photosensitizers with further enhancement under anoxic conditions. Oxygen is a strong triplet quencher and its removal increases triplet steady-state concentration. Consequently, a compound reactive towards triplets would decay faster under anoxic conditions. Previous photochemical studies with mafenamic acid also revealed increased photodegradation in the presence of a model photosensitizer, perinaphthenone, particularly under anoxic conditions. Here, the same trends were verified for all fenamates and diclofenac (Figure S15-S18).

Data in Figure 3B show reaction rate constants of compounds 1-5 under different experimental conditions, each normalized to the respective rate constant obtained in the presence of PN under air-saturated conditions, $ln(k/k_{PN})$. The normalized ratio, $ln(k/k_{PN})$, allows one to compare the effect of triplet quenchers on the overall photosensitization. The data demonstrate that anoxic conditions significantly increased the reaction rate constant by factor 3.5 to 5.7 (compared to degradation without sensitizer). Thus, compounds 1-5 significantly react with the triplet sensitizer. The reactivity with model sensitizers further reinforces the hypothesis that triplet state DOM may account for the additional
enhancement of photodegradation as observed for mefenamic acid and diclofenac. Data in Figure 3B further shows that the presence of a model antioxidant, caffeic acid, significantly quenched triplet sensitized degradation for compounds 1-5 by a factor of 1.2 to 3.2. Although caffeic acid also reacts with \(^3\)PN\(^*\), we estimated that this reaction only accounts for a minor change in the steady state concentration of \(^3\)PN\(^*\) (approx. 6%, Figure S6, Text S7). Flufenamic acid and meclofenamic acid show significant reactivity towards \(^3\)PN\(^*\) (Figure S15-S16), yet, a net quenching effect was observed in the presence of DOM in simulated sunlight, which may be attributed to reactions with antioxidant moieties of the DOM.

We hypothesized that the sensitizers react with these diphenylamine-based drugs by one-electron donation forming a radical intermediate that can be reduced back to the parent compound by electron donation from an antioxidant. These hypotheses were further investigated by transient absorption spectroscopy.

2.3.4 Radical Intermediates and Electron Transfer Properties. Laser flash photolysis experiments were conducted to further elucidate the reaction mechanism of diclofenac and fenamates with triplet sensitizers and antioxidants. First, the formation of radical intermediates upon reaction with the triplet sensitizer perinaphthenone, \(^3\)PN\(^*\), was demonstrated.

![Figure 4. Three-dimensional transient absorption spectra for (A) Triplet-excited perinaphthenone (\(^3\)PN\(^*\)) and (B) Mefenamic acid radical (MEF\(^*\)) after subtraction of scattered laser light. (C) Stern-Volmer plot shows the change in decay rate constant of \(^3\)PN\(^*\) as a function of mefenamic acid concentration to determine the bimolecular reaction rate constant, \(k(\text{MEF}^*)\). Samples were sparged with synthetic air during the experiment.]

As presented in Figure 4A, \(^3\)PN\(^*\) is a short-lived (1.7 µs in synthetic air) species with an absorbance centered around 479 nm. In the presence of \(^3\)PN\(^*\) and mefenamic acid an additional, longer-lived transient feature appeared with approximately 300 ns delay centered around 700 nm (Figure 4B). This feature was attributed to the mefenamic acid radical. Previously, the radical for unsubstituted diphenylamine was identified with a transient absorbance centered around 670 nm.\(^96\) Data in Figure 4C show the Stern-Volmer plot to determine the bimolecular reaction rate constant of \(^3\)PN\(^*\) with mefenamic acid, \(k(\text{MEF}^*)\), by monitoring the decay of the \(^3\)PN\(^*\) signal. The bimolecular reaction rate
constants of $^3PN^*$ with compounds 1-5 were determined accordingly and range from $20 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for mefenamic acid to $4.2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for diclofenac (Table 1, Figure S2-S5).

Lastly, the decelerating effect of antioxidants moieties within DOM towards the triplet reaction mechanism was investigated. Therefore, the decay rate of the radical intermediates of compound 1-4 were monitored in the presence of a model antioxidant. Here, lumichrome (LC) was used as the sensitizer because $^3\text{LC}^*$ has a higher triplet state one-electron reduction potential ($E^\circ = 1.91 \text{ eV}$) compared to $^3\text{PN}^*$ and more intense transient signals of the radicals were achieved, critical to evaluate their decay rates accurately.

Data in Figure 5 show the transient absorbance of the fenamate radicals as they appear in the presence of $^3\text{LC}^*$. The $^3\text{LC}^*$ feature (Figure S19), centered around 630 nm, reacts fast with the fenamates and is no longer visible on the selected timescale. In addition, for the reaction with all compounds 1-5, the transient absorbance of the lumichrome radical anion, ($\text{LC}^*$), centered around 425 nm (803 ns delay), was observed and agrees with its transient signal observed previously at a similar wavelength. The simultaneous occurrence of the sensitizer radical anion and the radicals of the test compounds strongly suggest that the reaction proceeds through a one electron transfer mechanism. Although the radical feature was not observed for diclofenac, the $\text{LC}^*$ was formed suggesting the same reaction mechanism.

Data in Figure 5F show the Stern-Volmer plot to determine the bimolecular reaction rate constants of Me$^*$ with a model antioxidant, ascorbic acid $k_{(\text{AA})}$, by monitoring the change in the decay rate constant of the Me$^*$ signal. The bimolecular reaction rate constants were determined accordingly for all fenamates and ranged from $62.1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for mefenamic acid to $3.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for meclofenamic acid (Table 1, Figures S7-S9).
Figure 5. Transient absorption spectra for the radical formation of (A) Mefenamic acid (Mef*), (B) Tolfenamic acid (Tol*), (C) Meclofenamic acid (Mec*), (D) Flufenamic acid (Flu*), (E) No radical formation for diclofenac, but triplet lumichrome (\(^3\)LC\(^*\)) has reacted and strong radical anion (LC\(^-\)) is formed. Blue vertical lines at 720 nm are a result of scattered laser light. (F) Stern-Volmer plot showing the decay rate constant of the Mef* versus the model antioxidant concentration (ascorbic acid) to determine the bimolecular reaction rate constant, \(k\) (AA). Samples were sparged with argon throughout the experiment.

Based on the high reactivity of compounds 1-5 with triplet sensitizer, one would expect an overall increase in photodegradation in the presence of DOM. However, DOM (PLFA) did not show net sensitizing effects in the solar simulator towards flufenamic acid and meclofenamic acid (summary of effects for compounds 1-5 shown in Table S4). First, the model sensitizer perinaphthenone employed here cannot represent all different sensitizing moieties within DOM. Perinaphthenone has a relatively low triplet state one-electron reduction potential (\(E^*_0 = 1.03\) V, SHE)\(^3\) and thus can be considered a conservative model compound for DOM triplets. Secondly, the effect of DOM varies with DOM source. Here, experiments with Suwannee River Fulvic Acid II (10 mg C L\(^-1\)) in simulated sunlight resulted in similar trends but slightly different decay rates than observed with PLFA (Figure S20). In addition, the high reactivities with model antioxidants demonstrate that redox-active DOM may decelerate photodegradation of fenamates effectively. Consequently, the presence of DOM may not always show a strong net sensitizing effect. These phenomena are particularly relevant for compounds reacting via a radical intermediate, which can be reduced by antioxidant moieties present in DOM. The overall effect of DOM on the photochemical half-lives of these compounds depends on the quantity and quality of redox-active moieties and varies among DOM sources.\(^{12}\)
2.4 Implications

The contribution of direct photochemical degradation varies among diclofenac and the fenamates and can play a dominant role at the water surface. While all compounds showed significant reactivity towards singlet oxygen and hydroxyl radicals, these reactive oxygen species can only partially contribute to their natural attenuation in the environment. Not all compounds showed a net increase of the photodegradation rate in the presence of DOM. One-electron oxidation and reduction mechanisms with sensitizing and antioxidant moieties within DOM have been positively identified by the radical intermediate detected in transient absorption spectroscopy. We demonstrate that DOM plays two roles in the photodegradation these diphenylamine-based drugs, which is similar to anilines, sulfonamide antibiotics, dimethylaniline-based drugs, and tryptophan. These compounds each undergo oxidation by triplet excited DOM, which proceeds through a radical intermediate that can be converted back to the parent compound by a suitable electron donor, such as antioxidant moieties in DOM. Consequently, the effect of DOM on the overall photodegradation rate of these compounds is dependent on the DOM’s redox properties.

While the presented data show that diclofenac and the fenamates can undergo direct and indirect photochemical reactions, the half-lives are relatively long when taking into account diurnal and seasonal sunlight intensities. Where natural attenuation may not curb the concentration of these NSAIDs enough, additional measures are required to either limit their input into surface waters or to enhance water treatment for drinking water purposes. Hollender et al. demonstrated that treatment using a primary clarification and ozonation was able to completely remove both mefenamic acid and diclofenac. Advanced oxidation technologies, such as ozonation, are however not implemented in most of the municipal wastewater treatment plants globally. In general diclofenac and mefenamic acid are more widely distributed and these compounds have been included in monitoring studies more regularly. Depending on the usage pattern of these pharmaceutical, the other fenamates should also be monitored as their common mode of action (cyclooxygenase inhibition) may result in mixture toxicity effects.

Acknowledgements

We gratefully acknowledge support of the Swiss National Science Foundation (Grant number 200021-156198). We thank Ladina Birolini for her support on this project.
2.5 References


95. Apak, R.; Guclu, K.; Demirata, B.; Ozyurek, M.; Celik, S. E.; Bektasoglu, B.; Berker, K. I.; Ozurt, D., Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules* 2007, **12**, (7), 1496-1547.


Chapter S2. Supplementary Information: Environmental Photochemistry of Fenamate NSAIDs and their Radical Intermediates

The supplementary information contains 20 figures, 4 tables, and details on methods for determination of the bimolecular reaction rate constant with $^1O_2$, Fraction of D$_2$O in solutions for KSIE experiments, calculation of steady-state concentrations of singlet oxygen and hydroxyl radicals, calculation of light screening factor, water column model, and calculation of $^3PN^*$ quenching caffeic acid (Text S1-S7).
Table S1. LogD_{ow} for Fenamates - Predicted with ACD/Labs software (www.chemspider.com).

<table>
<thead>
<tr>
<th>Fenamate Drug</th>
<th>LogD_{ow} (ionized, pH 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diclofenac</td>
<td>1.69</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>2.29</td>
</tr>
<tr>
<td>Meclofenamic acid</td>
<td>2.95</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>2.04</td>
</tr>
<tr>
<td>Tolfenamic acid</td>
<td>2.47</td>
</tr>
</tbody>
</table>

Text S1. Methods for determining the bimolecular rate constant with singlet oxygen.

Rose Bengal and visible light (> 455 nm)

This is a steady-state experiment in open borosilicate test tubes containing 40 µM FFA, 3 µM Rose Bengal as a $^{1}O_{2}$ source and 5 µM of the test compound. Samples were irradiated with a Xenon lamp using a 455 nm long-pass filter to inhibit any direct photolysis of the test compounds. Samples were taken at 0, 2.5, 5, 7.5, 10, 12.5, and 15 min for FFA quantification. Aliquots were taken at 0, 5, 10, 15, 20, 25, 30, and 40 min for the fenamate drugs. Dark controls of the samples kept in amber vials during the irradiation were also included. UV-vis absorbance measurements of samples at time 0 and 40 min were compared to determine the photobleaching of Rose Bengal during the experiment.

$^{1}O_{2}$ phosphorescence (laser spectroscopy)

Singlet oxygen phosphorescence was recorded at (1270 ± 5 nm) using a near-IR photo-multiplier tube detector. This configuration was built in our lab and modeled after the singlet oxygen phosphorescence detector described by Bilski et al\textsuperscript{103} and Jiménez-Banzo et al\textsuperscript{104}. Quartz cuvettes were filled with sensitizer in a solvent composition of 50:50, H\textsubscript{2}O:ACN. For these experiments 100 µM perinaphthenone and 5 µM Rose Bengal were used as sensitizers. A pump beam of 360 nm and 550 nm were used to excite PN and RB, respectively and their corresponding power was 80 and 70 mW. Increasing concentrations of fenamates were present in solution, 100 – 500 µM for flufenamic, mefenamic and tolfenamic acid, and 500 µM – 3000 µM for diclofenac and meclofenamic acid. The various fenamate concentrations also contained different amount of solvent (acetonitrile), which can also affect the lifetime of $^{1}O_{2}$, so controls were done with the same solvent composition, which did not contain fenamates to correct for this difference. The decay portion of the $^{1}O_{2}$ signal was fitted and represented the lifetime of the $^{1}O_{2}$. The decay rate ($k_{obs}$, s\textsuperscript{-1}) corresponds to 1/lifetime. The decay rate for each amount of fenamates added were plotted against each other in a Stern-Volmer plot. The data was collected using a software called TimeHarp. Data analysis was performed using Origin 9.1

Non-photochemical generation of $^{1}O_{2}$: H\textsubscript{2}O\textsubscript{2} decomposition with molybdate catalyst

Non-photochemical generation of $^{1}O_{2}$ was done using a method adapted from one used by Boreen et al\textsuperscript{4}. This experiment was carried out in amber sample vials that were covered to prevent photolysis. 50 µL of 1 mM H\textsubscript{2}O\textsubscript{2} was added to 4.95 mL of a carbonate (pH 10.0, 10 mM) solution containing 10 µM fenamate, 40 µM FFA, and 1 mM MoO\textsubscript{4}\textsuperscript{2-}. 375 µL aliquots were taken at 0, 10, 30, 60, 120, 180, and 360 min and added to 125 µL of sodium azide (507 mM) to quench the reaction. Samples were
analyzed with HPLC. Controls were done without MoO$_4^{2-}$ and without H$_2$O$_2$ to check whether fenamates were reactive with the catalyst or hydrogen peroxide.

![Diagram of Figure S1: Comparison of $k_{\text{rxn}}(1O_2,\text{fen})$ determined via different experimental methods. Black squares (■), and green circles (●), represent 1O$_2$ phosphorescence using perinaphthenone (PN) and Rose Bengal (RB) as a 1O$_2$ source, respectively. Blue triangles (▲) used chemical generation of 1O$_2$ with H$_2$O$_2$ and MoO$_4^{2-}$. Pink triangles (▼), show photochemical generation of 1O$_2$ with RB and light > 455 nm. Red diamonds (♦) represent kinetic solvent isotope effect (KSIE) experiments using PN as a 1O$_2$ source. * = no kinetic solvent isotope effect observed for diclofenac, so no rate constant could be determined.]

**Text S2. Composition of D$_2$O in KSIE experiments**

The fraction of D$_2$O in solution can be calculated using the following equation:

$$k_{\text{soln}} = \chi H_2O \cdot k_{H_2O} + \chi D_2O \cdot k_{D_2O}$$

with $k_{\text{soln}}$ being the observed reaction rate constant of the probe molecule furfuryl alcohol (FFA) in the solution tested, $k_{H_2O}$ being the observed reaction rate constant of FFA in 100% H$_2$O, and $k_{D_2O}$ being the reaction rate constant of FFA in 100% D$_2$O, as well as the unknown mole fractions of in H$_2$O and D$_2$O of the tested solution.

$$\chi H_2O = \frac{k_{\text{soln}} - k_{D_2O}}{k_{H_2O} - k_{D_2O}} \quad \chi D_2O = 1 - \chi H_2O$$

$k_{H_2O} = 2.54 \times 10^5$ s$^{-1}$; $k_{D_2O} = 1.79 \times 10^4$ s$^{-1}$, (based on lifetimes of 1O$_2$)$^{105}$

The calculated fractions of H$_2$O and D$_2$O in the experiments for all test compounds are listed in Table S2.
Table S2. Fraction of D$_2$O in solutions for KSIE experiments.

<table>
<thead>
<tr>
<th>Fenamate</th>
<th>Mole Fraction H$_2$O</th>
<th>Mole Fraction D$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diclofenac</td>
<td>9.8%</td>
<td>90.2%</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>11.3%</td>
<td>88.7%</td>
</tr>
<tr>
<td>Meclofenamic acid</td>
<td>18.0%</td>
<td>82.0%</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>13.8%</td>
<td>86.1%</td>
</tr>
<tr>
<td>Tolfenamic acid</td>
<td>21.6%</td>
<td>78.4%</td>
</tr>
</tbody>
</table>

Text S3. Calculation of steady-state concentration of singlet oxygen, [^1O$_2$]$_{ss}$

\[
[^1O_2]_{ss} = \frac{k_{obs}(FFA)}{k_{rxn,FFA}}
\]

$k_{obs} =$ observed degradation rate constant for FFA

\[
\ln k_{rxn,FFA} = -\left(\frac{1.59 \pm 0.06}{273.16 + T[^\circ C]}\right) + (23.82 \pm 0.21)
\]

T = temperature in degrees Celsius; see Appiani et al.$^5$

Text S4. Calculation of steady-state concentration of hydroxyl radical, [^*OH]$_{ss}$

\[
[^*OH]_{ss} = \frac{d[hTPA]/dt}{k_{rxn,TPA} \cdot [TPA] \cdot Y}
\]

$d[hTPA]/dt =$ rate of change for hTPA (in M$^{-1}$ s$^{-1}$)

[TPA] = initial concentration of TPA,

$k_{rxn,TPA} = 4.4 \times 10^9$ M$^{-1}$ s$^{-1}$, Y = 35% (production yield)$^6$
Stern-Volmer Plots for Calculating Reaction Rate Constant with triplet perinaphthenone - $k_{rxn(3PN)}$

**Figure S2.** Stern-Volmer Plot for determination of bimolecular reaction rate constant ($k_{rxn}$, M$^{-1}$s$^{-1}$) for the reaction between triplet perinaphthenone and diclofenac.

<table>
<thead>
<tr>
<th></th>
<th>slope</th>
<th>intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{rxn}$</td>
<td>$4.24 \pm 0.87 \times 10^8$ M$^{-1}$s$^{-1}$</td>
<td>$1.59 \pm 0.04 \times 10^6$ M$^{-1}$s$^{-1}$</td>
</tr>
<tr>
<td>Residual sum of squares</td>
<td>1.123</td>
<td></td>
</tr>
<tr>
<td>Adjusted R$^2$</td>
<td>0.849</td>
<td></td>
</tr>
</tbody>
</table>

**Figure S3.** Stern-Volmer Plot for determination of bimolecular reaction rate constant ($k_{rxn}$, M$^{-1}$s$^{-1}$) for the reaction between triplet perinaphthenone and flufenamic acid.

<table>
<thead>
<tr>
<th></th>
<th>slope</th>
<th>intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{rxn}$</td>
<td>$5.68 \pm 0.50 \times 10^8$ M$^{-1}$s$^{-1}$</td>
<td>$1.66 \pm 0.07 \times 10^6$ M$^{-1}$s$^{-1}$</td>
</tr>
<tr>
<td>Residual sum of squares</td>
<td>0.062</td>
<td></td>
</tr>
<tr>
<td>Adjusted R$^2$</td>
<td>0.969</td>
<td></td>
</tr>
</tbody>
</table>
Figure S4. Stern-Volmer Plot for determination of bimolecular reaction rate constant (\( k_{\text{rxn}} \), M\(^{-1}\) s\(^{-1}\)) for the reaction between triplet perinaphthenone and meclofenamic acid.

\[
\begin{array}{|c|c|}
\hline
k_{\text{rxn}} & 11.93 \pm 1.84 \times 10^8 \text{M}^{-1}\text{s}^{-1} \\
\hline
\text{intercept} & 1.53 \pm 0.06 \times 10^6 \text{M}^{-1}\text{s}^{-1} \\
\hline
\text{Residual sum of squares} & 0.642 \\
\hline
\text{Adjusted R}^2 & 0.910 \\
\hline
\end{array}
\]

Figure S5. Stern-Volmer Plot for determination of bimolecular reaction rate constant (\( k_{\text{rxn}} \), M\(^{-1}\) s\(^{-1}\)) for the reaction between triplet perinaphthenone and tolfenamic acid.

\[
\begin{array}{|c|c|}
\hline
k_{\text{rxn}} & 8.93 \pm 1.26 \times 10^8 \text{M}^{-1}\text{s}^{-1} \\
\hline
\text{intercept} & 1.78 \pm 0.05 \times 10^6 \text{M}^{-1}\text{s}^{-1} \\
\hline
\text{Residual sum of squares} & 1.413 \\
\hline
\text{Adjusted R}^2 & 0.924 \\
\hline
\end{array}
\]
Figure S6. Stern-Volmer Plot for determination of bimolecular reaction rate constant for the reaction between triplet perinaphthenone and caffeic acid.

Stern-Volmer Plots for Calculating Reaction Rate Constant with Antioxidant - $k_{\text{rxn}}$ (fen^−, ascorbic acid)

Table:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>slope</td>
<td>$3.53 \pm 1.26 \times 10^9 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>intercept</td>
<td>$0.50 \pm 0.08 \times 10^9 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>Residual sum of squares</td>
<td>0.395</td>
</tr>
<tr>
<td>Adjusted R²</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Figure S7. Stern-Volmer Plot for determination of bimolecular reaction rate constant ($k_{\text{rxn}}$, M$^{-1}$s$^{-1}$) for the reaction between flufenamic acid radical cation (Flu$^+$) and ascorbic acid.
Figure S8. Stern-Volmer Plot for determination of bimolecular reaction rate constant ($k_{rxn}$, M$^{-1}$s$^{-1}$) for the reaction between meclofenamic acid radical cation (Mec$^+$) and ascorbic acid.

Ascorbic acid (M) vs. Mec$^{••}$ decay rate constant (s$^{-1}$)

<table>
<thead>
<tr>
<th>$k_{rxn}$</th>
<th>slope</th>
<th>3.27 ± 0.61 x 10$^7$ M$^{-1}$s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>intercept</td>
<td>2.43 ± 0.33 x 10$^3$ M$^{-1}$s$^{-1}$</td>
</tr>
<tr>
<td>Residual sum of squares</td>
<td>0.213</td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.903</td>
<td></td>
</tr>
</tbody>
</table>

Figure S9. Stern-Volmer Plot for determination of bimolecular reaction rate constant ($k_{rxn}$, M$^{-1}$s$^{-1}$) for the reaction between tolfenamic acid radical cation (Tol$^{••}$) and ascorbic acid.

Ascorbic acid (M) vs. Tol$^{••}$ decay rate constant (s$^{-1}$)

<table>
<thead>
<tr>
<th>$k_{rxn}$</th>
<th>slope</th>
<th>2.06 ± 0.12 x 10$^8$ M$^{-1}$s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>intercept</td>
<td>7.01 ± 0.40 x 10$^3$ M$^{-1}$s$^{-1}$</td>
</tr>
<tr>
<td>Residual sum of squares</td>
<td>0.076</td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.993</td>
<td></td>
</tr>
</tbody>
</table>
Figure S10. Pseudo-first order degradation plots of the fenamate drugs in the solar simulator controlled at pH 7.5 for mefenamic acid (1, blue), tolfenamic acid (2, green), meclofenamic acid (3, red), flufenamic acid (4, purple), and diclofenac (5, black). The table inset describes their half-lives under experimental conditions.

Figure S11. Absorbance spectra for diclofenac and fenamates from 290 – 400 nm (left axis). The photo fluence rate for the solar simulator output is plotted on the right axis. Overlap of molar absorptivity peaks with solar simulator output peak represents light that can be absorbed by the molecules.
Text S5. Calculation of Light Screening Correction Factor.
Using Figure S above, decide the range of wavelengths where the light source and the test compound overlap. This range will be used to determine the screening correction factor based on method from Leifer et al.7.

First, calculate the “S-factor”.

\[ S = 1 - 10^{-az} \]

\[ a = \text{optical density at } \lambda, \text{ dependent on the absorption of the sensitizer; DOM.} \]
\[ z = \text{pathlength of light through test-tube} \]

Next calculate the intensity of light absorbed by the system, \(I_{t\lambda}\).

\[ I_{t\lambda} = \frac{S \cdot I_{0\lambda}}{\sum I_{0\lambda}} \]

\(I_{0\lambda}\) = incident light intensity of the light source, measured using a radiometer

Then, the amount of light that is transmitted (T) is calculated,

\[ T = \frac{I_{t\lambda}}{I_{0\lambda}} \]

Finally, from the transmission, it is possible to calculate the correction factor.

\[ CF = \frac{1}{T} \]

CF = correction factor

Table S3. Light Screening Correction Factor for diclofenac and fenamates in 10 mg L\(^{-1}\) PLFA to account for light attenuation from DOM.

<table>
<thead>
<tr>
<th>Fenamate Drug</th>
<th>Light Screening Correction Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diclofenac</td>
<td>1.15</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>1.09</td>
</tr>
<tr>
<td>Meclofenamic acid</td>
<td>1.10</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>1.08</td>
</tr>
<tr>
<td>Tolfenamic acid</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Table S4. Summary of contribution to indirect photodegradation from various photochemically produced reactive intermediates (PPRIs) under simulated sunlight with 10 mg C L\(^{-1}\) PLFA.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect of DOM (\Delta K_{obs}) (%)</th>
<th>(^1)O(_2) (%)</th>
<th>(^1)OH (%)</th>
<th>(^3)CDOM* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mefenamic acid</td>
<td>+95</td>
<td>24.0</td>
<td>1</td>
<td>71</td>
</tr>
<tr>
<td>Tolftenamic acid</td>
<td>+9</td>
<td>9.9</td>
<td>&gt;1</td>
<td>0</td>
</tr>
<tr>
<td>Meclofenamic acid</td>
<td>-14</td>
<td>4.0</td>
<td>&gt;1</td>
<td>n.a.(^a)</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>-2</td>
<td>1.4</td>
<td>&gt;1</td>
<td>n.a.(^a)</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>+19</td>
<td>0.0</td>
<td>&gt;1</td>
<td>19</td>
</tr>
</tbody>
</table>

\(^a\)Not analyzed because of overall net quenching effect of DOM
Text S7. Water Column Model (Figure 2b)
To estimate the change of direct and indirect photochemical degradation in a water column, the absorption spectrum of the organic matter solution and the test compounds was recorded and the light intensity of solar irradiation were used.

First, the wavelength dependent change of light intensity as a function of water depth $I_{\lambda, z}$ (mE cm$^{-2}$ s$^{-1}$), was calculated as:

$$I_{\lambda, z} = I_{\lambda, 0} \cdot 10^{-z \cdot a}$$

with $I_{\lambda, 0}$ being incident light intensity at the water surface, $a$ (cm) being the optical density at wavelength, $\lambda$, dependent on the absorption of the sensitizer; DOM, and $z$ (cm) being the water pathlength of light representing the water depth.

Second, the depth and wavelength dependent rate of light absorbance, $k_{a, z}$(mE cm$^{-3}$ s$^{-1}$), by the sensitizer DOM and the test compound were estimated as:

$$k_{a, z} = 2.303 \cdot a \cdot I_{\lambda, z}.$$

At each depth, the rates were summed across the wavelength spectrum where the DOM and test compounds absorb light within the solar spectrum, being 290-500 nm for DOM and 290-400 nm for diclofenac, mefenamic acid, tolfenamic acid, meclofenamic acid, and flufenamic acid.

The change in $k_{a, z}$ as a function of depth for DOM and test compounds is directly proportional to the relative decrease of indirect and direct photodegradation, respectively.

Figure S12. Light intensity of wavelengths 200-700 nm up to 1 m in the water column. Purple = 1 cm, blue = 10 cm, green = 20 cm, yellow = 30 cm, orange = 50 cm, and red = 1 m depth.
Figure S13. (KSIE) Pseudo-first order degradation of fenamates in 0.7 μM perinaphthenone; (A) diclofenac, (B) flufenamic acid, (C) meclofenamic acid, (D) mefenamic acid, (E) tolfenamic acid. Blue diamonds represent degradation in 90% D₂O and red hollow diamonds represent degradation in 100% H₂O. Panel F shows the KSIE (ratio of $k_{obs}$ (D₂O/H₂O)).

Figure S14. Competition Plots vs. pseudo-first order degradation of benzoic acid (BZA) and fenamates; (A) diclofenac, (B) flufenamic acid, (C) meclofenamic acid, (D) tolfenamic acid, and (E) mefenamic acid. Panel F shows the calculated bimolecular reaction rate constants for diclofenac and the fenamates.
Role of Triplet Sensitizing and Antioxidant moieties in DOM

Below are the remaining pseudo-first order degradation plots for flufenamic, meclofenamic, mefenamic, tolfenamic acid for the steady-state experiment with 0.77 µM perinaphthenone in air, argon sparged and with 10 µM caffeic acid.

**Figure S15.** Pseudo-first order degradation of flufenamic acid (5 µM) in enhanced UVA light in phosphate buffer (pH 7.5) only: black squares (■), in the presence of the triplet sensitizer perinaphthenone (PN, 0.7 µM): red circles (○), with PN and argon sparged: blue triangles (△), and with PN and the antioxidant caffeic acid (CA, 10 µM): green diamonds (◇), and the inset shows the reaction rate constants, \(k_{obs}\) and the log-normalised ratio of \(k_{obs}\), normalized to \(k_{obs}\) while sensitized with PN, \(\ln(k/k_{PN})\).

**Figure S16.** Pseudo-first order degradation of meclofenamic acid (5 µM) in enhanced UVA light in phosphate buffer (pH 7.5) only: black squares (■), in the presence of the triplet sensitizer perinaphthenone (PN, 0.7 µM): red circles (○), with PN and argon sparged: blue triangles (△), and with PN and the antioxidant caffeic acid (CA, 10 µM): green diamonds (◇), and the inset shows the reaction rate constants, \(k_{obs}\) and the log-normalised ratio of \(k_{obs}\), normalized to \(k_{obs}\) while sensitized with PN, \(\ln(k/k_{PN})\).
Figure S17. Pseudo-first order degradation of mefenamic acid (5 µM) in enhanced UVA light in phosphate buffer (pH 7.5) only: black squares (■), in the presence of the triplet sensitizer perinaphthenone (PN, 0.7 µM): red circles (●), with PN and argon sparged: blue triangles (▲), and with PN and the antioxidant caffeic acid (CA, 10 µM): green diamonds (◇), and the inset shows the reaction rate constants, $k_{obs}$ and the log-normalised ratio of $k_{obs}$, normalized to $k_{obs}$ while sensitized with PN, ln($k/k_{PN}$).

Figure S18. Pseudo-first order degradation of tolfenamic acid (5 µM) in enhanced UVA light in phosphate buffer (pH 7.5) only: black squares (■), in the presence of the triplet sensitizer perinaphthenone (PN, 0.7 µM): red circles (●), with PN and argon sparged: blue triangles (▲), and with PN and the antioxidant caffeic acid (CA, 10 µM): green diamonds (◇), and the inset shows the reaction rate constants, $k_{obs}$ and the log-normalised ratio of $k_{obs}$, normalized to $k_{obs}$ while sensitized with PN, ln($k/k_{PN}$).
Text S7. Calculation of $^3\text{PN}^*$ quenching by caffeic acid for steady-state experiments

The decay ($k_d$) of $^3\text{PN}^*$ in air sparged solution was calculated by the inverse its lifetime ($\tau = 1.7 \, \mu s$).

$$k_d = 5.8 \times 10^5 \, s^{-1}$$

The amount of triplet decay due to caffeic acid ($k_{CA}$) can be calculated by multiplying the bimolecular reaction rate constant of $k_{(^3\text{PN}^*)}$, (Figure S6) with the caffeic acid concentration for the steady-state experiment.

$$k_{CA} = (3.5 \times 10^9 \, M^{-1} s^{-1}) \times (10 \, \mu M)$$

$$k_{CA} = 3.5 \times 10^4 \, s^{-1}$$

$$Additional \, decay = \frac{k_d + k_{CA}}{k_d} = 6\%$$

Therefore, caffeic acid would contribute to 6% decay of $^3\text{PN}^*$ in solution.

Figure S19. Blank for triplet Lumichrome ($^3\text{LC}^*$). Pump beam = 360 nm. Sparged continuously with argon gas.
Figure S20. (A) Pseudo-first order degradation plots of the fenamate drugs in the solar simulator in solution with 10 mg.L\(^{-1}\) Suwannee River Fulvic Acid, controlled at pH 7.5, for mafenamic acid (1, blue), tolfenamic acid (2, green), flufenamic acid (4, purple), meclofenamic acid (3, red), and diclofenac (5, black). The table inset shows the half-lives under experimental conditions and the net change in reaction rate (\(\Delta k_{obs}\)) due to the DOM.

References


Chapter 3. Non-Singlet Oxygen Kinetic Solvent Isotope Effects in Aquatic Photochemistry

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Abstract

The kinetic solvent isotope effect (KSIE) is typically utilized in environmental photochemistry to elucidate whether a compound is susceptible to photooxidation by singlet oxygen (¹O₂), due to its known difference in lifetime in water (H₂O) versus heavy water (D₂O). Here, the overall indirect photodegradation rates of diarylamines in the presence of dissolved organic matter (DOM) were enhanced in D₂O to a greater extent than expected based on their reactivity with ¹O₂. For each diarylamine, the relative contribution of reaction with ¹O₂ to the observed KSIE was determined from high resolution data of ¹O₂ lifetimes by time-resolved infrared luminescence spectroscopy. The additional enhancement in D₂O beyond reaction with ¹O₂ contributed significantly to the observed KSIE for diarylamines (8-65%) and diclofenac (100%). The enhancement was ascribed to slower reduction of transient radical species of the diarylamines due to H/D exchange at DOM’s phenolic antioxidant moieties. A slower second-order reaction rate constant with a model antioxidant was verified for mefenamic acid radicals using transient absorption spectroscopy. Changes in lifetime and reactivity with triplet sensitizers were not responsible for the additional KSIE. Other pollutants with quenchable radical intermediates may also be susceptible to such an additional KSIE, which has to be considered when using the KSIE as a diagnostic tool.

Keywords: kinetic solvent isotope effect, singlet oxygen, triplet reactivity, proton-coupled electron transfer, deuterium isotope effects.
3.1 Introduction

Singlet oxygen \( (1^\text{O}_2) \) is a reactive oxygen species present in sunlit surface waters at steady-state concentrations ranging from \( 10^{-12} \) to \( 10^{-14} \) M.\textsuperscript{18, 19, 21, 106} \( 1^\text{O}_2 \) is formed through an energy transfer reaction between ground state oxygen \( (3^\text{O}_2) \) and photochemically produced excited triplet states of ubiquitous chromophoric dissolved organic matter (CDOM). Energy transfer to \( 3^\text{O}_2 \) occurs for most excited triplet states since the required energy to promote \( 3^\text{O}_2 \) to \( 1^\text{O}_2 \) is low \( (E_S = 94 \text{ kJ mol}^{-1}) \).\textsuperscript{3, 16, 17} The major reaction pathways of singlet oxygen include [2+2] and [2+4] cycloaddition, ene reactions, phenol and sulfide oxidation reactions.\textsuperscript{107} Thus, several organic compounds can be oxidized by \( 1^\text{O}_2 \), including cyclic dienes, polycyclic aromatic compounds, and heterocycles, as well as olefins containing allylic hydrogen atoms.\textsuperscript{4, 23, 24} Due to its specific reactivity with chemical probes, e.g., furfuryl alcohol,\textsuperscript{38, 108, 109} characterizing indirect photodegradation due to \( 1^\text{O}_2 \) has become a standard technique in the environmental photochemist’s toolbox. In addition, a kinetic solvent isotope effect (KSIE) has been relied upon as a diagnostic test to identify the reactivity of organic compounds with \( 1^\text{O}_2 \).\textsuperscript{5, 7, 9, 10, 18, 37, 110} This KSIE manifests itself in an acceleration of the observed degradation rate for a compound of interest when the solvent is changed from H\(_2\)O to D\(_2\)O. While the mechanisms of the KSIE for \( 1^\text{O}_2 \) are well understood, other photochemical transformation pathways could have their own solvent isotope effects and evidence for both is detailed below.

The major deactivation pathway of \( 1^\text{O}_2 \) results from energy transfer to the solvent and a KSIE for \( 1^\text{O}_2 \) results from a longer \( 1^\text{O}_2 \) lifetime in D\(_2\)O \( (67 \mu\text{s}) \)\textsuperscript{34} versus H\(_2\)O \( (3.6 \mu\text{s}) \).\textsuperscript{111} The vibrational frequencies of H\(_2\)O align with the electronically excited energy of \( 1^\text{O}_2 \) causing an efficient deactivation by transferring the correct quanta of energy. In D\(_2\)O, the vibrational frequencies are shifted and the energy transfer from \( 1^\text{O}_2 \) becomes less efficient and solvent deactivation slows down \( (k_d^\text{1O2} \text{ in Figure 1A}) \).\textsuperscript{112} As a consequence, in D\(_2\)O steady-state concentrations of \( 1^\text{O}_2 \) are higher, while the bimolecular reaction rate constants of \( 1^\text{O}_2 \) with compounds of interest are generally not affected.\textsuperscript{38}

In photochemistry, the diagnostic test to evaluate the reactivity of an organic pollutant with \( 1^\text{O}_2 \), relies on this \( 1^\text{O}_2 \)-specific KSIE. Therefore, the degradation rates of the compound of interest are measured in aqueous samples containing CDOM as a natural sensitizer, with and without enrichment of D\(_2\)O. The ratio of the rate constants in H\(_2\)O (unenriched) and in D\(_2\)O-enriched samples present the observed KSIE, \( (\text{KSIE}_{\text{obs}}) \), and is indicative of the contribution of \( 1^\text{O}_2 \). The method requires however, that neither second-order reaction rate constant of other oxidants with the compound of interest nor the oxidants’ steady-state concentrations change during D\(_2\)O-enrichment. Consequently, the method can be misleading for chemicals of interest that participate in non-\( 1^\text{O}_2 \) reactions that are D\(_2\)O-sensitive.

Other D\(_2\)O-sensitive reaction pathways may, however, contribute to the KSIE, including oxidation reactions by photochemically excited triplets and reduction reactions of radical intermediates. The
reaction with excited triplets may show a KSIE if deactivation of the triplet by the solvent and thereby the triplet steady-state concentration changes in D$_2$O ($k_{d3sens}^*$ in Figure 1A), e.g., due to altered vibrational coupling with the solvent, analogous to $^1$O$_2$. For example, the triplet lifetimes of methylene blue and substituted ruthenium(II) bipyridyl complexes are longer in D$_2$O versus H$_2$O. In addition the second-order reaction rate constant between excited triplets and the compound of interest could change depending on the solvent, which would also result in a KSIE ($k_{r3sens}^*$ in Figure 1B). Specifically, when the triplet mediated oxidation involves hydrogen abstraction from the compound of interest, an increase in the bond strength for isotopologues with deuterium substitution results in higher dissociation energies and potentially slower reactivity. Also, thermally induced reorganization of the solvent for outer sphere electron transfers can depend on solvent polarization, which determines the free activation energy, and thereby the reaction rate.

Figure 1. Schematic illustrating the processes that were investigated for a kinetic solvent isotope effect (KSIE). Panel (A) Lifetimes of oxidants: Excited triplet sensitizer ($^3sens^*$) and singlet oxygen ($^1$O$_2$). The $^3sens^*$ is produced when a chromophoric organic compound (i.e., sensitizer) absorbs a photon of light ($h\nu$) and $^1$O$_2$ is formed when the $^3sens^*$ transfers energy to molecular oxygen ($^3$O$_2$). The native lifetimes of the oxidants are inversely proportional to the solvent deactivation rates ($k_d$), which may be D$_2$O sensitive, as known for $^1$O$_2$. The native lifetimes of oxidants are independent of the compound of interest. Panel (B) Second-order reaction rate constants with the compound of interest, i.e., substituted diarylamines. The $^3sens^*$ reacts with diarylamines, producing a radical intermediate that can oxidize further ($k_2$). The radical intermediate can react with phenolic antioxidant moieties via a (proton-coupled) electron transfer (PCET) to regenerate the parent compound. H/D exchange with the solvent by the phenolic antioxidant may affect the second-order quenching rate constant ($k_{antiox}$) and exchange at reactive moieties of the $^3sens^*$ or at the compound of interest may affect the second-order oxidation rate constants ($k_{r3sens}^*$).

Another reaction that could exhibit a KSIE is the reduction of radical intermediates. Oxidation of several pollutants proceeds through radical intermediates, as observed for direct photochemical
ionization reaction of tryptophan\textsuperscript{116} and triplet sensitizer-mediated oxidation of anilines,\textsuperscript{11, 117, 118} sulfonamide antibiotics,\textsuperscript{15} beta blockers,\textsuperscript{110} and various pesticides, including chloroacetamide and phenylurea herbicides\textsuperscript{119} and finally diarylamines\textsuperscript{120} that we focus on herein. Reactive intermediates can be reduced back to parent compounds via an electron transfer (ET) or a proton-coupled electron transfer (PCET) pathway by a suitable reductant, also termed “antioxidant” hereafter. Phenolic moieties, known to be the major electron donating groups in DOM, are able to reduce radical intermediates and are readily susceptible to H/D exchange.\textsuperscript{12, 15, 117, 120} Accordingly, if the effectiveness for PCET of an antioxidant is decreased in D\textsubscript{2}O (k\textsubscript{antiox} in Figure 1B), the observed decay rate of a compound of interest in the presence of antioxidants would be enhanced in D\textsubscript{2}O.

3.2 Experimental Section

3.2.1 Materials and Solutions. Experiments were carried out in buffer from potassium phosphate dibasic (Sigma-Aldrich, \(\geq 98\%\)) and potassium dihydrogen phosphate (Fluka, \(\geq 99.5\%\)). Aqueous solutions were prepared with ultrapure water (>18 MΩ cm, Barnstead Nanopure Diamond system). The following reagents were purchased from Sigma-Aldrich and used as received: 3'-methoxyacetophenone (97%), acetonitrile (HPLC grade), caffeic acid (\(\geq 98\%\)), deuterium chloride solution (35 wt% in D\textsubscript{2}O, 99 atom% D), diclofenac sodium salt (\(\geq 98.5\%\)), lumi\textsuperscript{1}chrome, potassium deuterioxide solution (40 wt% solution in D\textsubscript{2}O, 98+ atom% D), sesamol (98%), sodium acetate trihydrate (\(\geq 99.0\%\)), superoxide dismutase from bovine erythrocytes (BioReagent \(\geq 3,000\) units/ mg protein), and tolfenamic acid. Dry acetonitrile was prepared using CaH\textsubscript{2} and stored under argon. Flufenamic acid (97%) was purchased from Acros Organics. Acetic acid (\(\geq 99.8\%\)) and mefenamic acid (\(\geq 98\%\)) were obtained from Fluka. Furfuryl alcohol (Merck, \(\geq 98\%\)) was distilled and kept under argon to avoid oxidation until use. Acetonitrile-d\textsubscript{3} (99 atom% D) and deuterium oxide (99.8 atom% D) were purchased from Armar Isotopes. Suwannee River Fulvic Acid (2S101F) was purchased from the International Humic Substance Society (IHSS, stock solutions at 85 mgC L\textsuperscript{-1} in H\textsubscript{2}O and D\textsubscript{2}O, were kept frozen until use). AlphaGaz 1 Ar (99.999% purity) and AlphaGaz 2 O\textsubscript{2} (99.9995% purity) were purchased from Carbagas AG.

3.2.2 Steady-State Photodegradation Experiments. The KSIE\textsubscript{obs} during photochemical degradation was investigated for five compounds, individually; diclofenac, mefenamic acid, tolfenamic acid, meclofenamic acid, and flufenamic acid. Photochemical experiments were conducted under three different conditions: phosphate buffered solution, in the presence of natural DOM, and in presence of a model sensitizer.

For tests with DOM, samples contained 5 µM of the compound of interest, 10 mgC L\textsuperscript{-1} SRFA, and 40 µM FFA buffered at pH/D 8 (phosphate buffer, 5 mM) in 0.81–0.94 (mole fraction, \(\chi\) D\textsubscript{2}O) or 1.00 (\(\chi\) H\textsubscript{2}O) (natural abundance of D\textsubscript{2}O approx. 0.015%)\textsuperscript{121}. The exact fraction of D\textsubscript{2}O was assessed with the
Chapter Three: Non-Singlet Oxygen Kinetic Solvent Isotope Effects in Aquatic Photochemistry

FFA probe (see data in SI, Table S1.) Samples were irradiated in open borosilicate test tubes with enhanced UVA light (12 bulbs, centered at 365 nm) on a turntable in a Rayonet photoreactor (Southern New England Ultraviolet Company, Branford, USA) with a polymer heat/bandpass filter situated between the lamps and the samples to remove light below 320 nm and also long wavelengths above 400 nm (269 LEE Heat Shield, Lee Filters, Hampshire, UK). Aliquots were taken over time in triplicate and analyzed for the compound of interest and FFA as described previously. Oxygen-free tests were performed in stoppered test tubes that were sparged with argon gas for 15 min prior to irradiation. To test for the impact of superoxide radical anion, 75 units mL\(^{-1}\) superoxide dismutase was added to the reaction mixture (diclofenac only).

For the test with model sensitizer, DOM was replaced by 0.77 µM perinaphthenone and the light exposure was reduced because kinetics proceeded faster due to higher triplet and \(^1\)O\(_2\) steady state concentrations (2 UVA bulbs). For diclofenac, an additional test was performed with model sensitizer and an antioxidant, 10 µM caffeic acid, present in the same vessel.

3.2.3 Data Analysis. The observed decay rate constants, \(k_{obs}\), were obtained by normalizing the concentration over the time course of the experiments to the respective initial concentration \((C_t/C_0)\), which were plotted in the log-normalized form, \(\ln(C_t/C_0)\) versus exposure time and fitted by a pseudo-first-order linear regression model where the slope represents \(k_{obs}\). The KSIE\(_{obs}\), was calculated as the ratio of \(k_{obs}\) in D\(_2\)O over \(k_{obs}\) in H\(_2\)O.

When the aqueous solvent is enriched with D\(_2\)O, the KSIE\(_{obs}\) can be a result of the superposition of multiple isotope effects from several processes. To evaluate the effect of other reaction pathways to the KSIE\(_{obs}\), we determined the contribution of \(^1\)O\(_2\) separately as KSIE\(_{1O2}\), and compared this to the overall KSIE\(_{obs}\). The KSIE\(_{1O2}\) for a compound if interest \((i)\) depends on the fraction of the overall observed decay that results from reaction with \(^1\)O\(_2\), \(f_{i,1O2}\):

\[
KSIE_{1O2} = KSIE_{FFA,1O2} \cdot f_{1O2}
\]

The KSIE\(_{1O2}\) of FFA, KSIE\(_{1O2, FFA}\) presents the maximum value with \(f_{1O2} = 1\). Values of \(f_{1O2}\) can be determined as:

\[
f_{1O2} = \frac{k_{1O2} \times [O_2]_{ss}}{k_{obs}}
\]

with the second-order reaction rate constant of the compound with \(^1\)O\(_2\), \(k_{1O2}\) (M\(^{-1}\) s\(^{-1}\)), the steady-state \(^1\)O\(_2\) concentrations, \([O_2]_{ss}\) (M), and the overall observed decay rate constants, \(k_{obs}\) (s\(^{-1}\)). The values of \(k_{1O2}\) for diarylamines were previously determined to range from 1.3-2.8 x 10\(^7\) M\(^{-1}\) s\(^{-1}\). These second-order reaction rate constants should not experience a KSIE, assuming that any H/D exchange in the molecule, e.g., at the amine functional group, is not affecting the reaction kinetics with \(^1\)O\(_2\).
The values for $[^1\text{O}_2]_{ss}$ were assessed by the kinetics of the probe molecule FFA, assuming that $f_{1\text{O}_2} = 1$ for FFA according to:

$$[^1\text{O}_2]_{ss} = \frac{k_{obs,\text{FFA}}}{k_{1\text{O}_2,\text{FFA}}}$$

(3)

with $k_{1\text{O}_2}$ of FFA determined as described previously.$^{38,120}$

The observed decay rate constant, $k_{obs}$, is equal to the sum of the individual pseudo-first order decay processes:

$$k_{obs} = k_{1\text{O}_2}[^1\text{O}_2]_{ss} + k_{direct} + k_{other}$$

(4)

where $k_{direct}$ is the apparent first-order rate constant for direct photodecay and $k_{other}$ is the observed decay rate constant by any other processes in addition to the contribution from $^1\text{O}_2$. The contribution of direct photodegradation, $k_{direct}$ to KSIE$_{obs}$ was assessed with additional photodegradation tests in the absence of sensitizer. The contribution of $k_{other}$ to KSIE$_{obs}$ was then calculated according to equation 4 from observed decay rate constants of the fenamates and diclofenac, $k_{obs}$. Mechanistically $k_{other}$ includes the reactions with triplet sensitizers and antioxidants, or other reductants as detailed in the SI (Text S1).

### 3.2.4 Transient Absorption Spectroscopy Experiments

Transient absorption spectroscopy was carried out using a pump-probe system (EOS, Ultrafast Systems, Sarasota, USA) with pump pulses produced by a regeneratively amplified Ti:sapphire laser, (output of 3.5 W at 795 nm, 1 kHz Solstice, Newport Spectra-Physics, Irvine, USA), and subsequent conversion to the desired wavelength using a TOPAS Optical Parametric Amplifier (Light Conversion, Vilnius, Lithuania) as previously described.$^{120}$ Excitation wavelengths for perinaphthenone, lumichrome, and 3-methoxyacetophenone were 370 nm, 400 nm, and 320 nm, respectively.

For triplet lifetime measurements, perinaphthenone, lumichrome, and 3-methoxyacetophenone (3-MAP) were tested as model sensitizers with no exchangeable protons, amine proton exchange, and enol tautomer proton exchange, respectively. Preliminary experiments to verify H/D exchange and required incubation times in D$_2$O were performed with mass spectrometry for lumichrome and $^1$H NMR and $^{13}$C $[^1$H] NMR for 3-MAP (details in Text S2 and Figures S1-S6).

To assess the triplet lifetimes, solutions were prepared with 100 µM triplet sensitizer in 50% dry acetonitrile and 50% H$_2$O or D$_2$O at pH/pD 8 (phosphate buffer) and pH/pD 13.6 for 3-MAP only. Samples were sparged for 4 minutes prior to, and during the measurement with either synthetic air or argon. The transient absorbance of the triplet signals was followed at 490 nm, 393 nm, and 550 nm
for perinaphthenone, lumichrome, and 3-MAP, respectively. The native triplet lifetimes were calculated by fitting the exponential decay of the respective delta absorption signals, ΔA.

Triplet reactivity was determined by adding increasing concentrations (50-1250 µM) of mefenamic acid to 100 µM lumichrome. The second-order reaction rate constant (k₃sens* in M⁻¹s⁻¹) was assessed as the slope of lumichrome triplet decay rate constants, (k₃,3sens* in s⁻¹, the inverse of its lifetime) versus mefenamic acid concentration in a Stern-Volmer plot. Here, lumichrome was chosen as a triplet sensitizer instead of perinaphthenone because its transient triplet signal was more resolved, free of overlapping signals. The triplet intensity (ΔA) of perinaphthenone and lumichrome showed a linear response with laser power from 1-3 µJ and 1-4 µJ, respectively (Figure S7).

Quenching of the radical intermediate was determined using 100 µM lumichrome, 400 µM mefenamic acid to generate the radical intermediate, and increasing concentrations of sesamol (25-400 µM), a natural phenolic compound, serving as a model antioxidant and quencher for this experiment. Sesamol is a preferred antioxidant for studying reduction of the radical intermediates because it has a low BDFE for the phenolic O–H bond (341.5 kJ/mol) to ensure quenching of the radical and contains only one proton that can exchange with the solvent, reaching maximum deuteration when dissolved in D₂O.⁴⁷ The second-order reaction rate constant of the mefenamic acid radical intermediate and antioxidant was derived from the slope of the radical decay rate constants, kₙ versus sesamol concentration in a Stern-Volmer plot. All data analysis of the transient experiments was performed using Surface Xplorer 4 (Ultrafast Systems, Sarasota, USA) and Origin Pro 9.0 (Origin Lab Corp. Northampton, MA).

3.2.5 Time-Resolved Infrared Luminescence Experiments. The tests to monitor ¹O₂ phosphorescence were performed as previously described³⁸,¹²⁰ with a regeneratively amplified laser (Solstice, Spectra-Physics, Darmstadt, Germany) which has a pulse width <100 fs, 1 kHz repetition rate. Excitation pulses were converted with a TOPAS optical parametric amplifier (Light Conversion, Vilnius, Lithuania) to the desired wavelength of 370 nm. The samples were prepared in triplicate and contained 100 µM riboflavin in varying compositions of D₂O (Table S2). The ¹O₂ phosphorescence was monitored 90° to the excitation, and the photons emitted passed through a 1270 ± 5 nm bandpass filter, before being detected with a near-IR PMT (Hamamatsu, model H10330-45). Samples were sparged with O₂ for 4 minutes before, and during the experiment. The ¹O₂ signal was fit to an exponential decay function to determine the lifetime under each condition. Singlet oxygen lifetime was independent of laser pulse energy (Figure S8).

3.3 Results and Discussion

3.3.1. KSIEobs from CDOM sensitized photodecay. Diclofenac and other diarylamines were selected to investigate evidence of isotope effects on reaction pathways beyond ¹O₂. We showed previously,
that diarylamines react with triplet state CDOM, and their indirect photochemistry proceeds through a radical intermediate that is quenchable by antioxidants. The triplet reactivity makes this group of compounds suitable for investigating potential KSIEs for a triplet-related reaction pathway.

Figure 2. Degradation kinetics during UVA irradiation with dissolved organic matter (DOM) as the triplet sensitizer (10 mg C L\(^{-1}\) Suwannee River Fulvic Acid, SRFA) in buffered aqueous solution (black circles) and enriched with D\(_2\)O (red squares) for furfuryl alcohol (panel A) and diclofenac (panel B). Panel C shows the degradation of diclofenac in DOM, under argon sparged conditions (anoxic). Respective mole fraction of D\(_2\)O (\(\chi\)) and pseudo-first order rate constants (\(k_{\text{obs}}\), simplified to \(k\)) in the figure) are presented. The KSIE\(_{\text{obs}}\) as the ratios of pseudo-first order rate constants, \(k_{\text{D}_2\text{O}}/k_{\text{H}_2\text{O}}\), are presented for experiments with model triplet sensitizer perinaphthenone (sens, 0.77 \(\mu\)M) and with DOM (SRFA, panel D). For diclofenac, additional experimental data with model sensitizer and the model antioxidant, caffeic acid (10 \(\mu\)M), is shown (sens + antiox.). The mole fraction of D\(_2\)O in solutions ranged from 0.81-0.94 (for data in panel D). Error bars represent one standard deviation.

Data in Figure 2 show the pseudo-first order photodecay in the presence of 10 mg C L\(^{-1}\) SRFA for the \(^1\)O\(_2\) probe molecule FFA (panel A) and diclofenac (panel B). The observed reaction rate constants (\(k_{\text{obs}},\ s^{-1}\)) demonstrate faster decay in heavy water. Enhanced degradation in D\(_2\)O was observed also for the remaining diarylamines (Figure S9). The KSIE\(_{\text{obs}}\) values are presented for exposure to model sensitizer (sens, 0.77 \(\mu\)M perinaphthenone) and organic matter sensitizer (DOM, 10 mg C L\(^{-1}\) SRFA, panel D). For all compounds, photodegradation was enhanced in D\(_2\)O, as all KSIE\(_{\text{obs}}\) values were larger than 1. This degradation rate enhancement upon D\(_2\)O-enrichment would traditionally be regarded as evidence that the compound of interest reacts with \(^1\)O\(_2\). Although most diarylamines show moderate reactivity toward \(^1\)O\(_2\) (\(k_{1O2} = 1.3 – 2.8 \times 10^7\ M^{-1}\ s^{-1}\)), diclofenac is known to be unreactive with \(^1\)O\(_2\). In accordance, only diclofenac showed negligible rate enhancement in D\(_2\)O in the presence of the model sensitizer as a source for \(^1\)O\(_2\), (sens: \(k_{\text{D}_2\text{O}}/k_{\text{H}_2\text{O}} = 1.12 \pm 0.14\)). However, a significant enhancement in D\(_2\)O was observed for diclofenac in the presence of CDOM (KSIE\(_{\text{obs}}= 1.73 \pm 0.09\), t-test, p < 0.05). Contrary to the system with model sensitizer, the CDOM also contains antioxidant moieties that are redox active. When a model antioxidant was added to the experiment with model
Chapter Three: Non-Singlet Oxygen Kinetic Solvent Isotope Effects in Aquatic Photochemistry

sensitizer, the decay for diclofenac was also accelerated in D₂O (Figure 2D: sens+antiox, KSIE_{obs}=1.28 ± 0.09, t-test, p < 0.05). To provide additional evidence that enhanced degradation in D₂O was not due to reaction with ¹O₂, a DOM-sensitized test was performed for diclofenac under argon saturated conditions (anoxic, Figure 2C) and the degradation was still enhanced in the D₂O enriched solution.

For all fenamates, the KSIE_{obs} was larger in the presence of CDOM as compared to the model sensitizer. These observations of a potential non-¹O₂-related KSIE in the presence of CDOM led to a detailed investigation to assess whether direct photodecay, reaction with triplet sensitizers, or the reaction of radical intermediates with reducing agents (i.e., antioxidants) were responsible. The fact that the KSIE_{obs} was larger in presence of DOM compared to the model sensitizer for all compounds, suggests that an additional reaction pathway other than ¹O₂ contributed to the rate enhancement in D₂O. We first determined the contribution of the reaction with ¹O₂ for each experiment with CDOM to then evaluate the magnitude of the additional KSIE.

3.3.2 Contribution of ¹O₂ pathway to KSIE_{obs}. The dependency of ¹O₂ lifetime on the amount of D₂O present needs to be considered when disentangling the different reaction pathways contributing to the overall KSIE_{obs} in steady-state experiments. While ¹O₂ lifetime, τ, has a well-known dependency on the solvent composition, some inconsistencies exist in the literature regarding the exact lifetimes.³³, ³⁴ We assessed the KSIE on the ¹O₂ lifetime by varying the fraction of D₂O from 0.0-1.0 and measuring decay of the ¹O₂ luminescence signal using state-of-the-art time-resolved infrared luminescence spectroscopy. Data in Figure 3 show the relationship between the lifetime of ¹O₂, KSIE_{¹O₂}(τ_{D₂O}/τ_{H₂O}) and the D₂O:H₂O solvent composition, χ_{D₂O} (see tabulated data in Table S2; Figure S10). The lifetimes in 100% H₂O and 100% D₂O were 3.56 µs and 63.68 µs, (solvent deactivation rates: k_{d}^{¹O₂}=2.81± 0.07 x 10⁵ s⁻¹ and 1.57 ± 0.02 x 10⁴ s⁻¹) respectively. The KSIE_{¹O₂} is high, with a maximum enhancement by factor of 17.9 from 100% H₂O to 100% D₂O. Consequently, the KSIE_{¹O₂} is very sensitive to the exact fraction of D₂O in experimental solutions. These values are in general agreement with the literature values compiled by Wilkinson et al. in the review of ¹O₂ lifetimes and decay rate constants in a multitude of solvents, ranging from 44 – 70 µs in D₂O (k_{d}^{¹O₂}=1.4 – 2.3 x 10⁴ s⁻¹) and 3.1 – 4.2 µs in H₂O (k_{d}^{¹O₂}=2.4 – 3.2 x 10⁵ s⁻¹).³³, ³⁴

The influence of ¹O₂ on KSIE_{obs} depends on the fraction of D₂O present and contribution of ¹O₂ to the overall degradation (f_{i,¹O₂}). Therefore, the fractions of D₂O were assessed with the KSIE_{obs} determined for FFA for which f_{i,¹O₂} = 1 and its decay kinetics are directly proportional to the ¹O₂ lifetime, which in turn is a function of the concentration of D₂O (details see Davis et al. 2017, Text S2).¹² In the steady-state experiments with DOM, the fraction of D₂O ranged from 0.81-0.94 (Table S1). These D₂O compositions result in maximum KSIE_{¹O₂} values ranging from 4.4-10 for compounds exclusively reacting with ¹O₂, like FFA (shaded bar at y-axis in Figure 3). Secondly, f_{i,¹O₂} was assessed for each
fenamate and diclofenac according to equation 2. The reactions with \(^1\)O\(_2\) contributed up to 19% for mefenamic acid and 8-16% for the other diarylamines to the overall decay, while no \(^1\)O\(_2\) contribution was observed for diclofenac \((f_{1,102} = 0.0)\). The insert in Figure 3 illustrates how the KSIE\(_{102}\) can vary for compounds with a different \(f_{1,102}\), shown for a fixed \(\chi\) D\(_2\)O of 0.90. The low \(f_{1,102}\) values, (< 0.2), for diarylamines explain why relatively low KSIE\(_{102}\) values can be expected from the reaction with \(^1\)O\(_2\) alone.

**Figure 3.** The Kinetic Solvent Isotope Effect, KSIE, as the ratio of singlet oxygen, \(^1\)O\(_2\), lifetimes in heavy over light water, \(\tau_{\text{D}_2\text{O}}/\tau_{\text{H}_2\text{O}}\), in relation to the fraction of D\(_2\)O in solution \((\chi\text{ D}_2\text{O})\). Data follows an inverse relationship: KSIE = \([-0.942\cdot(\chi\text{ D}_2\text{O}) + 0.988]\) as determined by time-resolved infrared luminescence spectroscopy with detection at 1270 nm. The shaded area under the curve indicates the range of D\(_2\)O (mole fraction, \(\chi\)) determined in the presented experiments using DOM as the triplet sensitizer (data in Figure 2). The inset shows, for a fixed \(\chi\) D\(_2\)O of 0.90, how the estimated KSIE\(_{102}\) decreases with the fraction of singlet oxygen-based decay of compound of interest \((f_{1,102})\) and when no other reaction contributes to the observed KSIE\(_{\text{obs}}\).

### 3.3.3 Contribution of non-\(^1\)O\(_2\) pathways to KSIE\(_{\text{obs}}\)

The overall observed degradation rate is the superposition of degradation resulting from several different pathways. To assess potential contribution of other reaction pathways to the KSIE\(_{\text{obs}}\), the estimated KSIE\(_{102}\) were subtracted from the KSIE\(_{\text{obs}}\) determined experimentally (data in Figure 2D). For most compounds, a significant contribution of a non-\(^1\)O\(_2\) related KSIE was evident.

Data in Figure 4 show the KSIE\(_{\text{obs}}\) measured in the presence of DOM (10 mgcL\(^{-1}\) SRFA) with the relative contribution of \(^1\)O\(_2\) and other degradation pathways (stacked bars above KSIE=1). The KSIE\(_{\text{obs}}\) values ranged from 1.7 to 2.4 but could only partially be attributed to the reaction with \(^1\)O\(_2\). The contribution of “other” processes to KSIE\(_{\text{obs}}\) was highest for diclofenac (100%), which does not react with \(^1\)O\(_2\) and ranged from 56-65% for mefenamic acid, fluatenamic acid, and tolenamic acid. The overall KSIE\(_{\text{obs}}\) for meclofenamic acid was 1.8, which can be attributed to at least 92% to the reaction with \(^1\)O\(_2\) and only a minor contribution from other processes (8%). This observation is supported by our previous finding, that, among these fenamates, meclofenamic acid has the greatest bimolecular reaction rate constant with \(^1\)O\(_2\) (2.8 x 10\(^7\) M\(^{-1}\)s\(^{-1}\)) and also exhibited the slowest bimolecular reaction
rate constants with the model antioxidant ascorbic acid \((3.3 \times 10^7 \text{ M}^{-1} \text{s}^{-1})\). Direct photochemical degradation contributed to the KSIE_{obs} only for flufenamic acid (Figure S11). Contrary to the other diarylamines studied, flufenamic acid is known to undergo direct photochemical transformation under experimental conditions (i.e., irradiance with 365 nm) by photohydrolysis of the trifluoromethyl group\(^{46}\), which evidently proceeds faster in D\(_2\)O. The rate of direct photodegradation is dependent on the rate of light absorbance by the compound of interest and the quantum yield for a transformation reaction. If one of these parameters changes in D\(_2\)O-enriched solution, direct phototransformation may contribute an additional KSIE. Absorbance spectra for all compounds can be found in the SI (Figure S12-S13). The mechanistic investigation of a KSIE from direct photochemical transformation pathways is compound specific and therefore beyond the scope of this work.

![Figure 4. The Kinetic Solvent Isotope Effect, KSIE, as the ratio of pseudo-first order degradation rate constants, in heavy over light water, \(k_{D_2O}/k_{H_2O}\). Black dashed horizontal line represents the threshold where no enhancement in D\(_2\)O is observed \((k_{D_2O}/k_{H_2O} = 1.0)\). The portion above 1.0 is broken down into contributing degradation pathways. White bars show the contribution from reaction with singlet oxygen, \(^1\text{O}_2\), ranging from 0-92%. The dark gray bar shows the contribution from direct photodegradation, only applying to flufenamic acid (Flu, 31%). Light gray bars show the contribution from other reaction pathways, ranging from 8-100%. Error bars represent one standard deviation.

In the following we demonstrate that the additional degradation enhancement in D\(_2\)O can most likely be attributed to the reduction of radical intermediates rather than the reaction with the triplet sensitizer (i.e., effect on triplet lifetime or reactivity).

### 3.3.4 KSIE from reduction reaction of radical intermediates

To investigate effect of reductants towards the KSIE_{obs}, we first investigated the isotope effect associated with a phenolic antioxidant, representative of phenolic DOM moieties. Additionally, we evaluated the role of superoxide as a reductant.

Radical intermediates of mefenamic acid were generated from the reaction with triplet excited lumichrome, \(^3\text{LC}^*\). Mefenamic radical decay was monitored in the presence of a phenolic antioxidant,
sesamol, in 100% H$_2$O and 100% D$_2$O by time-resolved transient absorption spectroscopy. Data in Figure 5 show increasing decay rate constants of the mefenamic acid radical intermediate with increasing concentrations sesamol. The second-order reaction rate constants, $k_{\text{antiox}}$, were assessed in H$_2$O and D$_2$O by linear regression. The experimental KSIE ($k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$) was 1.39 ± 0.23, consequently, the reactivity of the radical intermediate with the antioxidant was slightly slower in D$_2$O (t-test, p = 0.063). Sesamol has a relatively low BDFE, making it a strong antioxidant, leading to fast PCET. Previous studies investigating (PC)ETs reported lower isotope effects for compounds with higher reaction rate constants.$^{47,20}$ Reported isotope effects for the oxidation of phenol with humic and fulvic acids and triplet sensitizer, benzophenone were within a similar range (0.7-1.7).$^6$

While the absolute KSIE from the reaction of radical intermediates with antioxidants is relatively small, it was the only process in the hypothesized reactions scheme (Figure 1) that demonstrated a KSIE. The reduction of the radical intermediate back to the parent compound can only contribute significantly to the KSIE$_{\text{obs}}$ when this pathway is kinetically favourable. Consequently, the relative contribution of the antioxidant pathways is assumed to be high under the presented conditions. No KSIE was observed regarding the triplet lifetime and reactivity with model triplet sensitizers as detailed below.

Figure 5. Stern-Volmer plot of the decay rate constant of the mefenamic acid (Mef) radical signal in the presence of varying concentrations of sesamol (antioxidant) in light water (H$_2$O, black open circles) and in heavy water (D$_2$O, red filled squares). Shown are the second-order reaction rate constants, $k_{\text{antiox}}$, determined from linear regression and the p-value (t-test). Error bars represent one standard deviation.

Superoxide radical anions (O$_2^-$) are formed from electron transfer reactions from sensitizers to molecular oxygen and can not only act as oxidants (towards diphenols), or nucleophiles (towards typical SN2 substrates) but also as reductants (one-electron reducing agent in aprotic solutions) towards organic compounds.$^{122}$ Here, O$_2^-$ did not contribute significantly to the overall
photodegradation. The reaction kinetics of diclofenac in H\textsubscript{2}O showed no change when O\textsubscript{2}\textsuperscript{*} was quenched by superoxide dismutase, an enzyme that catalyzes the dismutation of superoxide radical anions to molecular oxygen and hydrogen peroxide (Figure S14-S15). However, an increased degradation was observed in the presence of O\textsubscript{2}\textsuperscript{*} quencher in 90\% D\textsubscript{2}O (k(withSOD)/k(withoutSOD) = 1.63 ±/\- 0.23, t-test, p< 0.05). We hypothesize that O\textsubscript{2}\textsuperscript{*} could also be formed through the reduction of ¹\textsuperscript{1}O\textsubscript{2}, in which case more would be produced under D\textsubscript{2}O enhanced conditions. Further tests to quantify the steady-state concentration O\textsubscript{2}\textsuperscript{*} would be needed to confirm this.

3.3.5 No KSIE on triplet lifetimes and reactivity. Three model triplet sensitizers were selected to investigate a potential KSIE on triplet lifetimes, τ. Lumichrome was selected as a model sensitizer for its potential H/D exchange at the amino protons and 3'-methoxyacetophenone for its potential keto-enol tautomerization chemistry (Figure S16). Perinaphthenone was selected as a model aromatic ketone with no exchangeable protons (negative control). First, ¹H NMR and ¹³C\{¹H\}NMR spectroscopy were performed for 3-MAP to confirm the exchange of deuterium from the solvent (see Figures S1-S4). The incorporation of deuterium into the 3-MAP was base-catalyzed and full incorporation only took place under highly basic conditions (pD = 13.6). High-resolution mass spectrometry was used to confirm the H/D exchange for lumichrome by the expected mass shift (see Figures S5-S6). No KSIE on the triplet lifetimes was observed under ambient (air-sparged) conditions (Figure S16, \(t_D/O/t_H/O = 1.04 \pm 0.03, 1.09 \pm 0.16, 1.04 \pm 0.03, \) and 1.02 ± 0.03 for lumichrome, 3-MAP pH/D = 8.0, 3-MAP pH/pD = 13.6 and perinaphthenone, respectively). A significant lifetime enhancement in D\textsubscript{2}O was only observed for 3-MAP under argon-sparged conditions, at basic pH/pD (\(t_D/0/0/t_H/O = 2.35 \pm 0.20\)). KSIE = 1.06 ± 0.03, 1.07 ± 0.15, 1.14 ± 0.04 for lumichrome, 3-MAP (pH/pD = 8.0) and perinaphthenone, under argon-sparged conditions, respectively.

The reactivity of excited state triplets was investigated as another potential source of a non-¹\textsuperscript{1}O\textsubscript{2} KSIE. Therefore, the second-order reaction rate constant, \(k^{3\text{ens}}\), between excited state lumichrome, \(^3\textit{LC}^*\), and mefenamic acid was determined by monitoring the decay rate constant of \(^3\textit{LC}^*\) in the presence of increasing concentrations of mefenamic acid as a quencher. No significant change in \(k^{3\text{ens}}\) was observed from 5.25 ± 0.27 M\textsuperscript{-1}s\textsuperscript{-1} in H\textsubscript{2}O to 4.66 ± 0.21 M\textsuperscript{-1}s\textsuperscript{-1} in D\textsubscript{2}O (t-test, p = 0.09, Figure S17). Consequently, the non-¹\textsuperscript{1}O\textsubscript{2} KSIE for photodecay of diarylamines and diclofenac is neither related to a change in triplet sensitizer lifetime nor its reactivity but is ascribed to the decelerated repair of the radical intermediates by antioxidant moieties in DOM.

3.4 Implications

In light of the findings of a non-¹\textsuperscript{1}O\textsubscript{2} related KSIE in environmental photochemical studies, we conclude that the KSIE method is not only a reasonable test for probing the reactivity of a compound with ¹O\textsubscript{2} but can even offer evidence for a quenchable radical intermediate step of the degradation pathway.
First, one should be mindful that changing the solvent can affect other reaction pathways, and not only the lifetime of $^1\text{O}_2$. Compounds that are oxidized by triplet excited molecules and transition through a radical intermediate are likely to exhibit an additional KSIE as demonstrated for diclofenac and diarylamines. Here, the additional KSIE is attributed to the reduction reaction of the radical intermediates with suitable antioxidant by a (PC)ET reaction. The likelihood that these reactions occur during environmental photochemistry is high, as oxidizing moieties in natural CDOM can act as the sensitizer and phenolic moieties in DOM can act as the antioxidant. While the absolute KSIE may be relatively small, we demonstrate that the process can contribute significantly to the overall KSIE when a repairable reaction with triplets is a dominant decay pathway. Comparing observed decay rate constants in D$_2$O and H$_2$O to determine second-order reaction rate constants with $^1\text{O}_2$ should only be used when one of the following criteria are fulfilled: (a) degradation does not proceed through a reducible radical intermediate, or (b) there are no reducing agents present in the solution. One should also include controls to determine potential effects of other pathways, e.g., direct photodegradation, towards the KSIE.

Acknowledgements

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3.5 References


Chapter S3. Supplementary Information: Non-Singlet Oxygen Kinetic Solvent Isotope Effects in Aquatic Photochemistry

The supplementary information contains 17 figures, 2 tables, and experimental details about the calculation of observed rate constants for singlet oxygen and other processes, as well as information about how the NMR samples were prepared in Text S1 and S2.
Table S1. Mole fraction ($\chi$) of D$_2$O in photochemical steady-state experiments.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>$\chi_{D_2O}$</th>
<th>$\chi_{H_2O}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mefenamic acid + SRFA</td>
<td>0.81</td>
<td>0.19</td>
</tr>
<tr>
<td>Tolfenamic acid + SRFA</td>
<td>0.86</td>
<td>0.14</td>
</tr>
<tr>
<td>Diclofenac + SRFA</td>
<td>0.94</td>
<td>0.06</td>
</tr>
<tr>
<td>Meclofenamic acid + SRFA</td>
<td>0.87</td>
<td>0.13</td>
</tr>
<tr>
<td>Flufenamic acid + SRFA</td>
<td>0.86</td>
<td>0.14</td>
</tr>
<tr>
<td>Mefenamic acid + buffer</td>
<td>0.99</td>
<td>0.01</td>
</tr>
<tr>
<td>Tolfenamic acid + buffer</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Diclofenac + buffer</td>
<td>0.93</td>
<td>0.07</td>
</tr>
<tr>
<td>Meclofenamic acid + buffer</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Flufenamic acid + buffer</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Mefenamic acid + buffer + SRFA</td>
<td>0.88</td>
<td>0.12</td>
</tr>
<tr>
<td>Tolfenamic acid + buffer + buffer</td>
<td>0.87</td>
<td>0.13</td>
</tr>
<tr>
<td>Meclofenamic acid + buffer + buffer</td>
<td>0.85</td>
<td>0.15</td>
</tr>
<tr>
<td>Flufenamic acid + buffer + buffer</td>
<td>0.91</td>
<td>0.09</td>
</tr>
<tr>
<td>Diclofenac + buffer + PN</td>
<td>0.91</td>
<td>0.05</td>
</tr>
<tr>
<td>Diclofenac + PN + Caffeic acid</td>
<td>0.88</td>
<td>0.12</td>
</tr>
<tr>
<td>Diclofenac + SRFA (argon sparged)</td>
<td>0.90</td>
<td>0.10</td>
</tr>
<tr>
<td>Diclofenac + SRFA + SOD</td>
<td>0.91</td>
<td>0.09</td>
</tr>
</tbody>
</table>

n.d. = not detected; no difference in the measured $k_{obs,FFA}$ ($k_{D_2O}/k_{H_2O}$)
SRFA = Suwannee River Fulvic Acid
PN = perinaphthenone
SOD = superoxide dismutase

**Text S1: Observed decay rate constant and “other” processes.**

The observed decay rate constant, $k_{obs}$, is equal to the sum of the individual pseudo-first order decay processes:

$$k_{obs} = k^{1O_2}_r\lbrack 1O_2\rbrack_{ss} + k_{direct} + k_{other} \quad \text{(S1)}$$

with $k_{direct}$ as the apparent first-order rate constant for direct photodecay and $k_{other}$ is the observed decay rate constant by any other processes in addition to the contribution from $1O_2$. The contribution of direct photodegradation, $k_{direct}$ to $KSIE_{obs}$ was assessed with additional photodegradation tests in the absence of sensitizer. The contribution of $k_{other}$ to $KSIE_{obs}$ was then calculated according to equation 4 from observed decay rate constants of the fenamates and diclofenac, $k_{obs}$. Mechanistically the observed decay rate constant $k_{other}$ includes the reactions with triplet sensitizers and antioxidants and can be expressed as:

$$k_{other} = \frac{k^{3sens}_r\lbrack 3sens\rbrack^*_{ss}\lbrack antiox\rbrack_{ss}k_2}{k^{3sens}_r\lbrack 3sens\rbrack^*_{ss}k_2 + k_2} \quad \text{(S2)}$$

with $k^{3sens}_r$ as the second-order rate constant for the reaction of diarylamines with triplets to form a diarylamine radical intermediate, $k_2$ is the first-order rate constant for further oxidation of the radical intermediate, and $k^{antiox}_r$ is the second-order rate constant for the reaction of a radical intermediate with antioxidant (antiox) to form the parent compound. Here, we investigated the respective second-order reaction rate constants for the reactions ($k^{3sens}_r$, $k^{antiox}_r$) as well as the first-order rate constants for the decay of $1O_2$ and $3sens^*$ determining their steady-state concentrations ($\lbrack 3sens\rbrack_{ss}$, $\lbrack antiox\rbrack_{ss}$) ($k^{1O_2}_r$, $k^{3sens}_r$) for a contribution to $KSIE_{obs}$ (compare Figure 1).
The reaction with hydroxyl radical was not considered to contribute to KSIE_{obs}, despite unspecific diffusion-controlled reactivity with many organic compounds due to extremely low steady-state concentrations (~10^{-17} \text{ M}) that are produced in sunlit waters.\textsuperscript{123,124,125} Such reactions may need to be revisited for other compounds of interest and experimental conditions (e.g., higher hydroxyl radical conditions).

Despite moderate steady-state concentrations (0.4-9.5 \times 10^{-9} \text{ M}) that are reported for sunlit waters\textsuperscript{123}, the reactions with organoperoxy radicals were not considered to contribute to KSIE_{obs}, because reported half-lives for an assortment of classes of organic chemicals (alkanes, olefins, benzyls, aldehydes, alcohols, and chlorocarbons) were very long, ranging from days to hundreds of years.\textsuperscript{2}

**Text S2: Preparation of samples for NMR analysis.**
All samples were prepared directly in NMR tubes for acidic, neutral and basic conditions and measured immediately after preparation.

For the acidic conditions, 0.5 µL 3-MAP and 2 µL DCl (35 wt. % in D in D_2O) were added to 50:50 v/v% D_2O and CD_3CN (acetonitrile-d_3) with a final volume of 500 µL. The final pH* was 1.15 (pD = 1.55) and the solution turned yellow upon mixing.

For the neutral conditions, 0.5 µL 3-MAP were added to 50:50 v/v% D_2O and CD_3CN (acetonitrile-d_3) with a final volume of 500 µL. The final pH* was 5.28 (pD = 5.68).

For the basic conditions, 0.5 µL 3-MAP and 2 µL KOD (40 wt. % in D_2O) were added to 50:50 v/v% D_2O and CD_3CN (acetonitrile-d_3) with a final volume of 500 µL. The final pH* was 13.95 (pD = 14.35). The spectra were recorded immediately after preparation and after 3 hours of incubation to allow enough time for proton-deuterium exchange.

Data were recorded with a 400 MHz (\textsuperscript{1}H NMR) and 100 MHz (\textsuperscript{13}C \{\textsuperscript{1}H\} NMR) with a Ascend Aeon\textsuperscript{TM} 400 NMR Spectrometer (Bruker, Billerica, Massachusetts, USA). Chemical shifts were referenced to corresponding residual solvent signals (CD_3CN: \textsuperscript{1}H NMR : δ_H = 1.94 ppm and \textsuperscript{13}C \{\textsuperscript{1}H\} NMR : δ_C =1.32 ppm using the software TopSpin.
Figure S1. 1H NMR of 3'-methoxyacetophenone for acidic conditions in 50:50 v/v% acetonitrile-d$_3$ and D$_2$O. The data obtained for neutral conditions were identical.

Figure S2. 1H NMR of 3'-methoxyacetophenone for basic conditions in 50:50 v/v% acetonitrile-d$_3$ and D$_2$O. Compared to neutral conditions, the peak at 2.5 ppm from the methoxy protons was no longer a strong singlet and was likely a mixture of the two partially deuterated species, indicated with the structures on the right.
Figure S3. $^1$H NMR of 3'-methoxyacetophenone for basic conditions after 3 hours incubation in 50:50 v/v% acetonitrile-$d_3$ and D$_2$O. Compared to the sample measured immediately, the peak at 2.5 ppm from the methoxy protons was no longer visible, which likely indicates complete deuteration, indicated with the structure on the right.

Figure S4. $^{13}$C ($^1$H) NMR of 3'-methoxyacetophenone for basic conditions after 3 hours incubation in 50:50 v/v% acetonitrile-$d_3$ and D$_2$O. A septet (m = 7, spin multiplicity) was visible at 26.5 ppm, which corresponds to a carbon with three D atoms. A pentet (m = 5) is visible at 26.5 ppm, corresponds to a carbon with two D atoms, indicated with the structures on the right.
Figure S5. Total Ion Chromatogram of lumichrome in 100% H2O at a concentration of 2 µM. Data was obtained with direct injection on a high-resolution electrospray ionization Orbitrap mass spectrometer (Thermo Exactive). The analysis was done in positive ionization mode at a spray voltage of 3.20 kV with a capillary temperature of 275 °C for a scan range of (238.0-248.0). The most dominant masses demonstrate that ionization of lumichrome (C_{12}H_{10}N_{4}O_{2}) formed a single charged species with a proton adduct (C_{12}H_{11}N_{4}O_{2}). The observed masses for [M+H]^+ were m/z = 243.0845 with relative abundance of 100 and m/z = 244.0908 with relative abundance of 9 for the two most abundant isotopologues, respectively.

Figure S6. Total Ion Chromatogram of lumichrome in 100% D2O at a concentration of 2 µM. Data was obtained with direct injection on a high-resolution electrospray ionization Orbitrap mass spectrometer (Thermo Exactive). The analysis was done in positive ionization mode at a spray voltage of 3.20 kV with a capillary temperature of 275 °C for a scan range of (238.0-248.0). The most abundant masses demonstrate that lumichrome was predominantly present with both amine protons exchanged with deuterium atoms (C_{12}H_{8}D_{2}N_{4}O_{2}) and ionization occurred largely with a deuterium adduct and to a minor extent with a proton adduct forming single charged species. For [M+D]^+ the observed masses were m/z = 246.1064 with relative abundance of 100 and m/z = 247.1098 with relative abundance of 8. For [M+H]^+ the observed masses were m/z = 245.1001 with relative abundance of 27.
Chapter Three: Supplementary Information

Figure S7. Effect of laser pulse energy (µJ) on triplet intensity (ΔA, mAu) for photosensitizers perinaphthenone (490 nm) and lumichrome (532 nm). Linear correlation demonstrates a monophotonic process. Stars indicate the pulse energy used for the transient absorption spectroscopy experiments (3.5 µJ). Gray symbols are excluded from linear fit due to saturation effects at higher laser intensities.

Figure S8. Effect of laser pulse energy (µJ) on $^1$O$_2$ lifetime (µs). Riboflavin was used as the photosensitizer to generate $^1$O$_2$ with a 370 nm excitation pulse and the $^1$O$_2$ lifetime was monitored at 1270 nm. The $^1$O$_2$ lifetime was independent of laser pulse energy in the tested range.
Figure S9. Degradation kinetics during UVA irradiation with dissolved organic matter (DOM) as the triplet sensitizer (10 mg·L⁻¹ Suwannee River Fulvic Acid, SRFA) in buffered aqueous solution (black circles) and enriched with D₂O (red squares) for flufenamic acid, meclofenamic acid, mefenamic acid, and tolfenamic acid in the presence of dissolved organic matter (10 mg·L⁻¹ SRFA). Black open circles represent conditions in H₂O and red filled circles in D₂O enriched solutions (χ = 0.81-0.87). The corresponding pseudo-first order rate constants, k, were assessed by linear regression.

Table S2. Effect of D₂O on singlet oxygen lifetime. The lifetimes (τ) and solvent deactivation rate constants (k_d,obs) of singlet oxygen was followed by time-resolved infrared luminescence monitoring phosphorescence at 1270 ± 5 nm at 20 ± 1°C. Data is presented as the average ± one standard deviation from triplicate measurements.

<table>
<thead>
<tr>
<th>Mol H₂O</th>
<th>Mol D₂O</th>
<th>χ D₂O</th>
<th>τ (µs)</th>
<th>k_d,obs x 10⁴ (s⁻¹)</th>
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<td>0.555</td>
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<td>0.00</td>
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<td>28.1 ± 0.70</td>
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<tr>
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Figure S10. Effect of D$_2$O on singlet oxygen lifetime plotted as the solvent deactivation rate constants ($k_{d,obs}$) for the $^1$O$_2$ signal versus the mole fraction of D$_2$O in solution.

Figure S11. Influence of D$_2$O enrichment on direct photodegradation kinetics of diclofenac, flufenamic acid, meclofenamic acid, mefenamic acid, and tolfenamic acid during UVA irradiation in the absence of a sensitizer in phosphate buffered solutions (5 mM, pH 7). Black open circles represent conditions in H$_2$O and red filled circles in D$_2$O enriched solutions (93-99%). The corresponding pseudo-first order rate constants, $k$, were assessed by linear regression.
Chapter Three: Supplementary Information

Figure S12. UV-vis absorbance spectra for fenamates (A – diclofenac, B – flufenamic acid, C – meclofenamic acid, D – mefenamic acid, E – tolfenamic acid) in 100% H₂O (black circles) and 100% D₂O (red squares).

Figure S13. UV-vis absorbance spectra for antioxidants (A – sesamol, B – caffeic acid), photosensitizers (C – 3-methoxyacetophenone pH 8 & pH 13, D – perinaphthenone, E – lumichrome), and DOM (F – Suwannee River Fulvic Acid).
Figure S14. Effect of superoxide dismutase (SOD, 75 units mL\(^{-1}\)) on the photodegradation kinetics of diclofenac in 10 mg C L\(^{-1}\) Suwannee River Fulvic Acid DOM in UVA light in 100% H\(_2\)O (panel A) and 90% D\(_2\)O (panel B). The corresponding pseudo-first order rate constants, \(k\), were assessed by linear regression.

Figure S15. Effect of superoxide dismutase (SOD, 75 units mL\(^{-1}\)) on the photodegradation kinetics of furfuryl alcohol (FFA) simultaneously monitored with diclofenac in 10 mg C L\(^{-1}\) Suwannee River Fulvic Acid DOM in UVA light in 100% H\(_2\)O (panel A) and 90% D\(_2\)O (panel B).
A) Amino protons - Example: lumichrome

\[ \text{Figure S16. No effect on the lifetime of triplets was assessed for three examples of triplet sensitizers used to represent potential moieties in CDOM. Exchangeable protons shown in red. Observed lifetimes, } \tau, \text{ from transient absorption spectroscopy in } H_2O \text{ and } D_2O \text{ for air-sparged and argon-sparged conditions are shown below their structures. On the right, the calculated kinetic solvent isotope effect (KSIE) is presented.} \]

B) Enol tautomer - Example: 3'-methoxyacetophenone

\[ \text{Figure S17. No KSIE on reactivity of triplet sensitizer was assessed by Stern-Volmer plot of the decay rate constants of the lumichrome triplet (} ^3LC^* \text{) signal versus mefenamic acid concentration in } H_2O \text{ (black open circles) and in } D_2O \text{ (red filled circles).} \]
References


Chapter 4. Fate Processes of Antimicrobial Peptides Daptomycin, Bacitracin, and Polymyxins in Environmental Systems

This chapter has been accepted by:

Environment International

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Abstract

Antimicrobial peptides (AMPs) are increasingly important as a last resort against multi-drug resistant bacteria due to resistance formation towards conventional antibiotics. However, many AMPs were introduced to the market before environmental risk assessment was required, e.g., by the European Medicines Agency (EMA) since 1998. While AMPs have been administered as antibiotics and growth promoters in feedstock since the 1960s and were reconsidered for human medicine by the EMA in 2013, details about their mobility and persistence in the environment remain unknown. This study investigated the environmental fate of three commonly used AMPs: bacitracins, daptomycin, and polymyxins B and E (Colistin). We observed moderate sorption affinity of daptomycin to standard European soils (K_d=20.6-48.6), while polymyxins adsorbed irreversibly. Bacitracin variants sorbed slightly to sandy soil (K_d=5.8-8) and significantly to clayey soil (K_d=169-250). We further investigated photochemical and microbial transformation processes relevant in surface waters. We demonstrated that phototransformation of all AMPs was enhanced in the presence of dissolved organic matter and fast bimolecular reaction rate constant with singlet oxygen contributed largely to indirect phototransformation (15-41%). Phototransformation product analysis for daptomycin was consistent with expected modifications of the tryptophan and kynurenine moieties. Moreover, riverine biofilm communities demonstrated biotransformation potential for all AMPs. Our findings of sorption behaviour, photo- and biotransformation suggest that these processes play a critical role in the fate of bacitracins, daptomycin, and polymyxins in environmental systems.

Keywords: nonribosomal peptides, colistin, sorption, phototransformation, biotransformation, antibiotics
4.1 Introduction

Antimicrobial peptides (AMPs) are oligopeptide-based antibiotics with a wide spectrum of target organisms ranging from multi-drug resistant bacteria to viruses. The AMPs can be naturally produced by prokaryotes (e.g., bacteria) and eukaryotes (e.g. protozoan, fungi, plants, insects, and animals). Daptomycin, bacitracins, and polymyxins (including colistin) present three AMP classes that are utilized as pharmaceuticals in human and veterinary medicine and are further investigated in the present work. Daptomycin is produced by *Streptomycetes roseosporus* (actinobacteria), bacitracins by *Bacillus subtillis*, and polymyxins by *Paenibacillus polymyxa*. While daptomycin has been used as an antibiotic only since the late 1990s, bacitracins and the polymyxins were discovered and used for their antibiotic properties since nearly half a century earlier. Until recently, the therapeutic use of these AMPs in human medicine has been limited to topical application due to reports of severe nephrotoxicity (i.e., effect on kidney function). However, as a result of resistance formation toward conventional antibiotics, AMPs became increasingly important as a last resort towards multi-drug resistant bacteria. In 2011, the World Health Organization reclassified colistin and daptomycin as critically important antimicrobials for human medicine because they are the sole or one of a limited available therapy to treat serious human diseases and they are also used to treat diseases caused by organisms that may be transmitted to humans from non-human sources. As is the case for many pharmaceuticals, not all of the administered dose of AMPs is resorbed in the patient, and without systematic metabolism, significant amounts are excreted in their unaltered bioactive forms. Excretion from human patients and subsequent incomplete removal during wastewater treatment is, however, not the only route for AMPs to enter the environment. Each of the AMPs under consideration have been employed in veterinary medicine. In particular bacitracins and colistin (i.e., polymyxin E) have been administered as antibiotics and growth promoters in the feedstock industry since the 1960s. Thus, application of animal manure to agricultural fields presents another critical point source of AMPs.

While a global awareness for resistance formation towards conventional antibiotics exists, AMPs were thought to be less prone to resistance formation, until recently. One common mode of action of AMPs is the interference with the cell membrane of gram-positive (e.g., by daptomycin, bacitracins) and gram-negative (e.g., by polymyxins) bacteria. Since a modification of the general structure of the membrane appeared less likely, resistance was not expected to be readily acquired (e.g., horizontal gene transfer), which recently proved to be a wrong assumption. The first colistin-resistant *Escherichia coli* was reported in 2016 in a slaughtered pig from an intensive farm near Shanghai and was also present in 15% of tested retail meat and in 1% of retrospectively analyzed samples of human hospital patients from 2011-2014. Since the colistin resistance gene *mcr-1* was discovered, additional genes *mcr-2* to *mcr-9* were identified in recent years. These genes have been found to cause a modification of the lipid A in lipopolysaccharide (LPS) membranes. In 2018, these resistance genes...
were identified in pig manure, human, and environmental samples, not only in Asian countries but also in Australia, Europe, Africa, North America and South America. 

Understanding the environmental fate of AMPs is extremely important, considering the biological activity of the AMPs and the risk of spreading antimicrobial resistance. Bacitracins and polymyxins were introduced to the market before rigorous environmental risk assessment for anthropogenic chemicals was required (e.g. by the European Medicines Agency since 1998). Today, we lack essential information about environmental concentrations and the behaviour of these AMPs. In 2015, a usage pattern-based exposure screening model was used to predict average and worst-case scenario environmental concentrations of veterinary antibiotics. Within this prediction model, colistin was forecasted to have average soil concentrations of roughly 100 (µg kg⁻¹ dw) from chicken, pig, and cattle manure combined. These numbers do not consider degradation rates of colistin in the environment because no studies on environmental fate processes were reported for polymyxins to that date. In 2017, the EMA reported on the phase I environmental risk assessment for daptomycin. The predicted environmental concentration (PEC) of daptomycin in surface water was estimated to range from 3.6 µg/L to 0.48 µg/L, considering dose administered to adults and children, respectively. Even if the drug is only administered for 14 days, the yearly PECs (0.08 µg/L) would exceed the action limit of 0.01 µg/L, therefore necessitating phase II environmental risk assessment. The parameters required by the phase II risk assessment regarding partitioning between environmental phases and transformation processes are essential to determine the mobility and stability of AMPs (e.g., octanol/water partitioning by OECD test guideline 107, adsorption – desorption by OECD test guideline 106, ready biodegradability by OECD test guideline 301). The AMPs considered in this study are large molecules (MW > 1000 g/mol), and consist of a cyclic peptide component, connected to either a linear peptide chain (in bacitracins), an aliphatic chains (in polymyxins) or a combination of both (in daptomycin). The AMPs are non-ribosomal peptides, which means they contain not only the standard amino acids, but also other non-proteinogenic building blocks. These compounds are biosynthesized via several post-translational modifications, which results in mixtures comprising similar variants with small changes to the overall common core molecular structure (Figure ). All investigated AMPs are amphiphilic and ionic at environmentally relevant pHs.

Herein, we present first empirical data for these AMPs regarding their sorption behaviour to selected European standardized soils, their photochemical transformation processes in surface waters, and their biodegradability in the presence of riverine biofilm suspensions.
4.2 Materials and Methods

**4.2.1 Chemicals & Solutions.** Aqueous solutions were prepared with ultrapure water (>18 MΩ cm, Barnstead Nanopure Diamond system). The following reagents were all purchased from TCI Chemicals Europe (Zwijndrecht, Belgium) and used as received: Colistin Sulfate (mixture) (>90.0% colistin A + B, Lot DTY3C-CF), Daptomycin (>94.0%, Lot TAL2L-Q0), Polymyxin B Sulfate (9-12% phenylalanine; max 10% water, Lot IJ3PM-LP). The following reagents were all purchased from Sigma-Aldrich (Buchs, Switzerland) and used as received: calcium chloride dihydrate (ACS reagent ≥99%), perinaphthenone (97%), sodium acetate trihydrate (BioUltra ≥ 99.5%), sodium benzoate (≥99.0%), and bacitracin zinc salt from Bacillus licheniformis (~70,000 U/g, Lot # 1421481V) was purchased from Sigma Life Sciences. Deuterium oxide (99.8 atom% D) was purchase from Armar Isotopes (Döttingen, Switzerland). Furfuryl alcohol (Merck, ≥98%) was distilled prior to use and kept under argon to prevent oxidation. Acetonitrile (Optima ® LC-MS grade) was purchased from Fischer Chemicals. Suwannee River Fulvic Acid (SRFA, 2S101F) was purchased from the International Humic Substance Society. Standard soil types 2.1 (sand) and 6S (clay) were purchased from LUFA Speyer and had the following soil characteristics: Soil 2.1: pH (0.01 M CaCl₂) 4.70 ± 0.01; 2.8 ± 0.7% clay content; and Soil 6S: pH (0.01 M CaCl₂) 6.88 ± 0.02; 41.7 ± 1.1% clay content. Because preliminary
testing revealed that the polymyxins sorb partially to plastic materials, only glassware was used and contact with plastic, including vial lids and pipette tips, was avoided whenever possible.

**4.2.2 Sorption Experiments.** The experimental procedure was adapted from the OECD 106 guideline: Adsorption-Desorption Using a Batch Equilibrium Method. Two standardized European soils were selected, LUFA 2.1 and LUFA 6S, which differ in native pH, organic carbon content, cation exchange capacity and soil texture (details in Table S1, S2). The soils were sieved to a particle size ≤2 mm. Soil was added to the sorption set-ups gravimetrically, and aliquots were dried overnight at 105°C to determine the dry weight of the samples (Table S3).

First, adsorption kinetics for one concentration of each of the test substances were assessed to determine the optimal contact time for the adsorption equilibrium (Figure S1). Sacrificial samples (parallel method) were prepared, where 50 mg of soil and 1 mL of 10 mM CaCl₂ were added in duplicate vials for each kinetic sampling point and placed on horizontal shaker (250 rpm). After 12 hours, 10 µM of the test compound was spiked and vials were removed after 0, 4, 8, 24 and 30 h. Samples were centrifuged directly in the HPLC vial (5 min, 4000xg) using a micro-centrifuge (Centrifuge 5427 R, Eppendorf AG, Hamburg). Supernatant (300 µL) was transferred (conical glass inserts inside a plastic centrifuge tube) and centrifuged again (10 min, 10 000xg). This supernatant (250 µL) was stored at -20°C before analysis with LC/HRMS (details below). Additional samples were prepared to verify the stability of test compounds in the dissolved phase of the soil supernatant to account for potential losses due to native soil pH, extractable extracellular enzymes and sorption to the test vessels. For these tests, soil was in contact with CaCl₂ solution for 12 h before the supernatant was separated from the solids (centrifugation), spiked with test compounds, and AMP concentrations were monitored. Adsorption isotherm tests were performed using the same set-up with varied concentrations of the test compounds in triplicate ranging from 1-100 µM and an incubation time of 6 h, which proved sufficient to achieve equilibrium.

**4.2.3 Phototransformation in Sunlight.** Test compounds (10 µM) were exposed to simulated sunlight (Heraeus model Suntest CPS+) in open quartz test tubes, positioned at a 20° angle from the horizontal plane, 30 cm below the light source, and submerged in a temperature-controlled water bath (21°C ± 1°C). Furfuryl alcohol (FFA, 40 µM) was added to each test tube for the quantification of singlet oxygen. Direct photolysis experiments were performed in 5 mM phosphate buffer at pH 7.5. Quantum yield calculations were performed for daptomycin for the range of 290 to 400 nm as described previously.148 The contribution of indirect phototransformation was tested in the presence of 10 mg C L⁻¹ dissolved organic carbon (SRFA). During light exposure, aliquots were removed from the test tube in triplicate and analysed as described below. Dark controls were included.
4.2.3.1 Reactivity with Singlet Oxygen. Daptomycin, polymyxin B, and polymyxin E were tested for their reactivity with singlet oxygen, \( (^1\text{O}_2) \). Test compound (10 µM), the \(^1\text{O}_2\) photosensitizer perinaphthenone (0.77 µM), and \(^1\text{O}_2\) probe FFA (40 µM) were added to a solution of either H\(_2\)O or D\(_2\)O (approximately 90%). Samples were irradiated with UVA light centered around 365 nm on a merry-go-around Rayonet photoreactor, and aliquots were removed during exposure. The bimolecular reaction rate constant with \(^1\text{O}_2\) was determined based on known the kinetic solvent isotope effect for \(^1\text{O}_2\) lifetimes as previously described.\(^{148}\) Bacitracin’s reactivity with \(^1\text{O}_2\) was not included here because it has previously been studied.\(^{52}\) None of the AMPs underwent significant direct photodegradation under UVA irradiation during these experiments.

4.2.3.2 Transient Absorption Spectroscopy. We investigated further that the observed kinetic solvent isotope effect in the \(^1\text{O}_2\) test with polymyxins did not result from a reaction with triplet excited state of perinaphthenone (\(^3\text{PN}^*\)) that we used as the \(^1\text{O}_2\) photosensitizer. Therefore, transient absorption spectroscopy was used to monitor the lifetime of \(^3\text{PN}^*\). Experiments were performed in H\(_2\)O and D\(_2\)O to determine whether a potential reaction with the triplet occurred and whether a kinetic solvent isotope effect was apparent. Transient absorption spectroscopy was carried out using a pump-probe system (EOS, Ultrafast Systems, Sarasota, USA). Pump pulses were produced by a regeneratively amplified Ti:sapphire laser, (output of 3.5 W at 795 nm, 1 kHz Solstice, Newport Spectra-Physics, Irvine, USA), which were converted to the desired excitation wavelength of 370 nm using a TOPAS Optical Parametric Amplifier (Light Conversion, Vilnius, Lithuania). Samples contained 100 µM perinaphthenone and 300 µM polymyxin E or B (due to aqueous solubility limits) and were sparged with a gas mixture of 5% O\(_2\) with 95% N\(_2\), starting 5 minutes before irradiation. The time-dependent change in absorbance (ΔA) for the triplet-excited state feature (\(^3\text{PN}^*\), averaged from 479-495 nm) was monitored. Transient absorbance traces were fit to an exponential decay function for lifetime estimates, \(t (= 1/k_{\text{obs}})\) (OriginPro 9.0, OriginLab Corp. Northampton, MA).

2.4 Biotransformation. The experimental procedure was adapted from the OECD guideline 309: Aerobic Mineralisation in Surface Water-Simulation Biodegradation Test. Periphyton biofilms were grown on glass slides supplied with water from a local stream in flow-channels in May-June 2018 (average water temperature 17ºC), and July-August 2018 (average water temperature 18ºC) as previously described.\(^{149}\) Channels were operated at a constant flow rate of 0.34 m·s\(^{-1}\) under a light/dark cycle of 12:12 h with BioSun fluorescent tubes (MLT Moderne Licht-Technik AG, Wettingen, Switzerland). After 4 weeks, the established biofilm was removed from glass slides, suspended in stream water and filtered (0.95 µm nylon net) to remove larger periphyton agglomerations and small organisms. For the sorption control experiments, the filtered suspension was subjected to a freeze-thaw cycle and autoclaved (120ºC) and this process was repeated for one experiment, as indicated below. The pH of the suspension ranged from 7.7 to 8.1 and ash-free/dry weights were measured.
Chapter Four: Fate Processes of Antimicrobial Peptides Daptomycin, Bacitracin, and Polymyxins in Environmental Systems

For the biotransformation test, 40 mL of suspensions were placed into 100 mL Schott bottles, spiked with AMPs from aqueous stock solutions with duplicate bottles for each AMP (0.2 µM for daptomycin, 2 µM for polymyxins and bacitracins). Sodium benzoate (10 µM) was added to each bottle as a readily degradable reference substance. Bottles were closed with cotton plugs, placed on a horizontal shaker table (22 rpm) at a room temperature of 19.6 ± 1.0°C and exposed to the same light conditions used during biofilm colonization. In addition, a UV-light filter (226, LEE filters, Hampshire, UK) was placed between the samples and the light source to avoid direct photochemical transformation. Aliquots were taken after the AMPs were spiked (triplicates) and every 24 hours thereafter (duplicates) from each bottle for up to 7 days. These aliquots were centrifuged in glass tubes and the supernatant was frozen at -20°C until further analysis. To exclude effects of the subsampling, AMPs were also spiked to 1 mL and 5 mL suspensions in individual vials (in duplicate) using the parallel method and at intermediate times complete vials were sacrificed and processed as described for the aliquots above. To control for abiotic transformation (hydrolysis, phototransformation), AMPs were also spiked into ultrapure water and sampled in a similar manner as described above. In addition, daptomycin and bacitracin were monitored for 30 hours in autoclaved biofilm and filtered (0.45 µm) autoclaved biofilm to examine initial sorption to biofilm material or hydrolysis in the cell-free dissolved phase of the biofilm, respectively.

2.5 Sample Analysis. The samples were analyzed for AMPs by high performance liquid chromatography with high resolution tandem mass spectrometry (HPLC/HRMS/MS). Samples (10 µL) were injected onto a solid-core C18 column (Agilent InfinityLab Poroshell 120 EC-C18, 3.0 x 100mm; 2.7(µm) with eluent A (0.1% formic acid in ultrapure water) and eluent B (0.1% formic acid in 100% acetonitrile) with a flow rate of 0.35 mL·min⁻¹ (gradient methods in Tables S5). After chromatographic separation, AMPs were detected with a high resolution Orbitrap mass spectrometer (Fusion Lumos, Thermo Fisher Scientific) equipped with a heated electron spray ionization (H-ESI) source. The ion source was operated at a spray voltage of 3000 V in the positive ion mode, ion transfer tube temperature of 275°C, vaporizer temperature of 250°C, sheath gas of 15 (arbitrary units, Arb), auxiliary gas of 10 (Arb), and sweep gas of 1 (Arb). Data was acquired in full scan mode with a m/z range: 500 – 1650, resolution: 240,000 (at 200 m/z), maximum injection time: 50 ms, automatic gain control (AGC) target: 1.0x10⁵ and data dependent MS² (ddMS²) was collected with HCD collision energy: 35%, Orbitrap detection at 15,000 resolution, from 80 m/z, AGC target: 5.0x10⁴, maximum injection time: 22 ms. With each sequence, external calibrations were measured in ultrapure water and respective matrix (soil/biofilm suspension or buffer) to account for matrix effects (Figure S2).

Analysis of benzoic acid and FFA was performed with HPLC and absorbance detection (Ultimate 3000, Dionex) on a C18 column (Waters XBridge C18, 2.1x50 mm; 3.5µm), with an injection volume of 20 µL, and 1 mL min⁻¹ flow rate. Benzoic acid was detected with an isocratic eluent ratio of 70:30
(A: 0.1% formic acid in ultrapure water; B: 0.1% formic acid in 100% acetonitrile), by absorption at 245 nm. Furfuryl alcohol was detected with an isocratic eluent ratio of 90:10 (A: sodium acetate buffer, pH 5.9, 15.6 mM; B: 100% acetonitrile), by absorption at 219 nm.

2.6 Data Analysis. Concentrations of AMPs were quantified using their [M+2H]^{2+} charge states (daptomycin m/z = 810.8625, polymyxin B1 m/z = 602.3822, polymyxin B2 m/z = 595.3744, polymyxin E1 m/z = 585.3901, polymyxin E2 m/z = 578.3822, bacitracin A1 m/z = 711.8817, bacitracin B1 m/z = 704.8739, bacitracin C1 m/z = 697.8661) with Skyline (version 4.2). Limits of quantification were defined as ten times the standard deviation of the y-intercept, divided by the slope (of the linear range of the calibration curve); ranging from 0.01-4.7 mg/L for daptomycin, 0.5-9.4 mg/L for polymyxin Bs, 0.9-11.7 mg/L for polymyxin Es, 0.6-9.7 mg/L for bacitracin A1, 0.3-5.6 mg/L for bacitracin B1 and 0.07-1.3 mg/L for bacitracin C1 (Text S1.). Observed degradation rate constants (k, s⁻¹) were derived by linear regression as the slope from log-normalized concentration of the parent compound divided by the mean of the initial concentration, ln(C/C₀) over exposure time. Possible transformation products were prominent m/z values observed in the total ion chromatogram, which grew in during the kinetic time series. In addition, suspect screening for previously reported transformation products was performed (Table S6). For solutions in D₂O from the experiment to derive the bimolecular O₂ reaction rate constants, H/D exchange of amino protons occurred and changes in the m/z were considered in the analysis (details in Text S2). To demonstrate that the O₂ method based on the kinetic solvent isotope effect is reproducible and that the data analysis does not introduce any artefacts from the mass spectrometry analysis in the presence of H/D exchange, the experiment was repeated for polymyxin E with a different analytical method. Here, the samples were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) prior to analysis by HPLC with fluorescence detection (details in Text S3, Figure S3).

4.3 Results and Discussion

4.3.1 Sorption of Antimicrobial Peptides to Soil.

To evaluate the ability of AMPs to sorb to the terrestrial soil or suspended particles, adsorption coefficients (K₄ values) were determined for two standard soils. Data in Figure 2 show the AMP concentrations measured in the aqueous phase and the calculated concentrations in the soils. The K₄ values were derived as the slope of the linear range of these soil adsorption isotherms. The two soils were selected because of their different characteristics: LUFA 2.1 is silty sand with pH 4.70, and 0.71% organic carbon and LUFA 6S is clayey loam with pH 6.88 and 1.77% organic carbon. Clay minerals present in the soil, (i.e. silicates, etc), give soils a permanent negative surface charge. Organic surfaces, (i.e. humic organic matter), have a variable surface charge and depend on the protonation state of ionisable functional groups (i.e. carboxylic –COOH, or phenolic –OH). Daptomycin is anionic at these pH values and the K₄ values ranged from 20 to 40 L kg⁻¹ between the soils (Table 1, Figure
2). By normalizing the $K_d$ values by the soil organic carbon content, the $K_{oc}$ values closely agreed for both soils (LUFA 2.1: 2903 ± 310; LUFA 6S: 2745 ± 197). Adsorption of anionic surfactants is known to mainly be influenced by hydrophobic mechanisms, supporting a strong correlation with soil organic matter content.\textsuperscript{151} While our data suggests that the sorption behaviour of daptomycin is likely dependent on the soil organic carbon content, we recognize that recent sorption theory challenges this simplified concept when sorption is governed by processes other than hydrophobic interactions.\textsuperscript{152, 153}

The $K_d$ values for the bacitracins were similar across the different variants but differed strongly between the soils, with 5.8-8 L kg\textsuperscript{-1} for LUFA 2.1 and $K_d = 169$\textsuperscript{2}250 L kg\textsuperscript{-1} for LUFA 6S. Normalization with organic carbon content did not account for the difference in $K_d$ values. In the sandy, more acidic LUFA 2.1 soil, all bacitracin variants remained predominantly in the aqueous phase (i.e., lower $K_d$ values) and the values increased by two orders of magnitude for the clayey, pH neutral LUFA 6S soil. At the pH values tested, both carboxylic acid moieties in bacitracin are expected to be deprotonated and thus negatively charged, while the two primary amines are protonated and thus positively charged. Consequently, the weaker cationic character may explain the more moderate sorption behaviour of bacitracins compared to polymyxins. The pKa value of the histidine moiety in bacitracin, ($pK_a \sim 6.9$)\textsuperscript{26}, lies between the pH of the tested soils. Consequently, protonated histidine (His-H\textsuperscript{+}) may decrease the affinity to the more acidic LUFA 2.1 soil. Soil pH and clay content of soil affected the adsorption behaviour of bacitracin variants to a greater extent than soil organic carbon content. Our observations agree with previous adsorption studies for bacitracin that observed variations of sorbed amounts by more than one order of magnitude between a soil and montmorillonite clay.\textsuperscript{53, 54}

For polymyxins, strong sorption was observed and relatively high LOQs did not allow the quantification of the remaining concentration in the aqueous phase in most samples. In addition, no quantitative extraction from the soil with various solvents (i.e., acetonitrile, methanol, acetone, ethyl acetate) could be achieved. Only the highest concentration spiked for the sorption isotherm for polymyxin E could be quantified after incubation with LUFA 2.1 and the $K_d$ values based on 1-point of the isotherm curve suggest strong sorption for polymyxin E1 (111 ± 36 L kg\textsuperscript{-1}) and E2 (256 ± 116 L kg\textsuperscript{-1}). A minimum limit of the $K_d$ for polymyxin E1 and E2 to LUFA 6S was estimated based on the limit of quantification and also suggests strong sorption (Table 1). We verified that the disappearance of polymyxins from the aqueous phase was not due to sorption to the glass or equipment used in the sampling procedure. Because we observed that polymyxins have a high tendency to sorb to plastic materials including polytetrafluoroethene (PTFE, TEFLO\textsuperscript{TM}), only glassware was used in the experimental procedures presented. We verified that neither abiotic hydrolysis nor soil-derived dissolved organic matter, including potential extracted extracellular enzymes contributed to the observed loss of polymyxins from solution with control incubations in filtered soil supernatant, in which polymyxins were stable. At
environmental conditions, with pHs below 9, polymyxins are cationic surfactants, which tend to adsorb strongly to clays, colloidal materials, and organic matter and estimates of US EPA’s EPI Suite using the Molecular Connectivity Index Methods predict a log $K_{oc}$ value of 8.7, supporting our observations for polymyxins. A recent study demonstrated that the bioavailability of polymyxin to act as an antimicrobial is significantly reduced upon sorption to soil.

Figure 2. Soil adsorption isotherms for (A) daptomycin, (B) bacitracin A (circles), bacitracin B (triangles), and bacitracin C (squares) in standardized European soils LUFA 2.1 (red) and 6S (grey).

In the absence of empirical data, models used to predict environmental partitioning mostly rely on calculated octanol-water partitioning coefficients ($K_{ow}$ values), which range over up to 10 log units for daptomycin (-0.47 to -9.77), polymyxin B (-0.89 to -7.2), polymyxin E (0.22-6.83) and bacitracin A (0.8 to -7.25), depending on the model type (Table S7). While the limitations of using $K_{ow}$ values for the predictions of environmental partitioning, especially for ionic compounds, are known, $K_{ow}$ values are still widely used when no empirical data exists. Currently, risk assessment benefits from model predictions of expected environmental concentrations the empirical data on soil partitioning coefficients presented here can be applied in such calculations for the AMPs. In the future, testing different soils and soil chemistry parameters systematically would further define the variability of sorption behaviour of the AMPs in different environments.

While all tested AMPs showed susceptibility to sorb to soil material, daptomycin and bacitracins in particular can remain in solution at significant proportions, depending on the soil type. In the following, we also evaluated the phototransformation and biotransformation processes of daptomycin, bacitracins, and polymyxins once they reach surface waters.
Table 1. Overview of experimentally derived parameters: adsorption coefficients for two standard soils, abiotic degradation rate constants from the solar simulator for direct and indirect photochemical transformation, and bimolecular reaction rate constant with \( ^1O_2 \).

<table>
<thead>
<tr>
<th>AMPs</th>
<th>Adsorption</th>
<th>Simulated Sunlight</th>
<th>Singlet Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_a )</td>
<td>( K_d )</td>
<td>( k_{obs} )</td>
</tr>
<tr>
<td></td>
<td>(L kg(^{-1}))</td>
<td>(L kg(^{-1}))</td>
<td>(direct) (x 10(^{10}) s(^{-1}))</td>
</tr>
<tr>
<td>Bacitracin A</td>
<td>8 ± 2</td>
<td>250 ± 50</td>
<td>n.d.</td>
</tr>
<tr>
<td>Bacitracin B</td>
<td>6 ± 2</td>
<td>169 ± 33</td>
<td>n.d.</td>
</tr>
<tr>
<td>Bacitracin C</td>
<td>5.8 ± 0.5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>20.6 ± 0.8</td>
<td>40 ± 2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Polymyxin B1</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Polymyxin B2</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Polymyxin E1</td>
<td>111 ± 36°</td>
<td>&gt; 52°</td>
<td>n.d.</td>
</tr>
<tr>
<td>Polymyxin E2</td>
<td>256 ± 116°</td>
<td>&gt; 185°</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\( ^1O_2 \): literature values reported in Lundeen et al. 2016; \( ^1O_2 \): rate constant for indirect phototransformation only (direct \( k_{obs} \) subtracted from the total \( k_{obs} \); determined using only 1 point from the isotherm; \( ^2 \): minimum \( K_a \) value based on spiked concentration and the limit of quantification for the experiment; n.a. = not analysed, n.d. = analysed but not detected; abbreviations: LC = lumichrome, RB = Rose Bengal, PN = perinaphthenone.

4.3.2 Phototransformation The AMPs were exposed to simulated sunlight to determine the contribution of direct and indirect phototransformation by photochemically produced reactive intermediates (PPRIs) generated from chromophoric dissolved organic matter (10 mg\(_C\)L\(^{-1}\) SRFA). Only daptomycin underwent direct phototransformation, with a low quantum yield (\( \Phi = 0.0003 \)). Daptomycin consists of two chromophoric residues, i.e., tryptophan and kynurenine, and absorbs light in the UV range of the solar spectrum. (Figure S4, UV-vis spectra of AMPs and light spectrum of solar simulator). Data in Figure 3 show that all AMPs underwent indirect phototransformation in the presence of organic matter, which indicates reactivity with PPRIs. The polymyxins reacted significantly faster than daptomycin and bacitracins. While hydroxyl radicals are highly reactive PPRIs, the steady state concentration was determined to be low, with 2 \( \times \) 10\(^{-17}\) M during the solar simulator experiments (using terephthalic acid as a probe molecule, data not shown). This is comparable to environmental concentrations reported for surface waters.\(^{155}\) Assuming diffusion controlled bimolecular reaction rate constants (\(~1\times10^9\) M\(^{-1}\)s\(^{-1}\)) we can estimate that hydroxyl radicals may only contribute less than 1% to the observed indirect phototransformation rate of the AMPs. In the following, the reaction with the PPRI singlet oxygen (\(^1O_2\)) is presented in more detail.

4.3.2.1. Reaction with Singlet Oxygen. The \(^1O_2\) steady-state concentrations during the solar simulator experiment ranged from 1.8-3.8 \( \times \) 10\(^{-13}\) M (10 mg\(_C\)L\(^{-1}\) SRFA) and these concentrations are representative for sunlit surface waters (10\(^{-14}\) -10\(^{-12}\) M\(^{-1}\))\(^{18, 19, 21, 106}\). The bimolecular reaction rate constants with \(^1O_2\) (\( k_{obs} \), M\(^{-1}\)s\(^{-1}\)) were determined by rate comparison in H\(_2\)O and D\(_2\)O; taking advantage of the kinetic solvent isotope effect, which makes \(^1O_2\) longer lived in D\(_2\)O and thus causes faster observed degradation in D\(_2\)O (Figure S3 and S5)\(^{93, 156}\). All AMPs tested showed significant reactivity with \(^1O_2\) (Table 1). The reaction of \(^1O_2\) with bacitracin A (5.2-6.2 \( \times \) 10\(^{-7}\) M\(^{-1}\)s\(^{-1}\)) has been determined.
previously and can be explained by the reactive histidine residues \((7.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}\) at neutral pH\)\(^{52}\). The bimolecular reaction rate constant for daptomycin with \(^1\text{O}_2\) was \(1.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}\) and thereby one order of magnitude slower than for polymyxins (10.2-14.7 \(\times 10^7 \text{ M}^{-1} \text{ s}^{-1}\)). With that, the relative contribution to the overall loss during exposure to simulated sunlight was 15\(\pm\)4\% for daptomycin, 43\(\pm\)4\% for bacitracins, 41\(\pm\)14\% for polymyxins B1 and B2, 29\(\pm\)8\% for polymyxin E1, and 40\(\pm\)11\% for polymyxin E2. We conclude that the reaction with \(^1\text{O}_2\) can contribute significantly to the indirect phototransformation of all AMPs in sunlit surface waters.

### 4.3.2.2. Phototransformation products

To investigate the reaction mechanisms, we further evaluated the observable transformation products for daptomycin and polymyxins. The daptomycin moieties that are reactive towards \(^1\text{O}_2\) are tryptophan and kynurenine\(^{158-160}\). The bimolecular reaction rate constant of freely dissolved tryptophan \((3.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1})\) is by factor three higher than the observed rate for daptomycin\(^{26}\) and the integration in the secondary peptide structure may affect its reactivity\(^{158-160}\). We tentatively identified transformation products of daptomycin from the reaction with \(^1\text{O}_2\) based on accurate mass, isotope pattern, diagnostic evidence from the secondary fragmentation and kinetic formation patterns. We observed several products as a result of known transformation reactions of tryptophan and kynurenine with \(^1\text{O}_2\), being (di-)hydroxylated moieties \(([+16], [+32])\), tryptophan dione \(([+30])\), N-formylkynurenine \(([+32])\) or a combination of hydroxylated kynurenine with tryptophan dione \(([+46])\), dihydroxy tryptophan dione \(([+60])\), and dihydroxy N-formylkynurenine \(([+80], \text{Table A8})\)\(^{158-160}\). The kinetic data analysis demonstrates that several products were formed immediately upon parent transformation \(([+14], [+30], [+32], [+46])\) and decayed again with ongoing exposure to \(^1\text{O}_2\), while more highly oxygenated transformation products \([+60]\) and \([+80]\) were formed slower over the course of the experiment (Figure S6).

For polymyxins, the high reactivity with \(^1\text{O}_2\) was not expected because no intuitive target can be identified within the molecular structure. The observed kinetic solvent isotope effect was significant and reproducible in two independent experiments and analysed by two different analytical quantification methods (Text S3, Figure S3). To exclude non-\(^1\text{O}_2\) reaction pathways that could contribute to the decay of polymyxins we conducted controls for abiotic hydrolysis, direct photochemistry, and reaction with the ground state and triplet state sensitizer used to produce \(^1\text{O}_2\). Hydrolysis and direct phototransformation can be excluded as dark controls and controls without \(^1\text{O}_2\) sensitizer in UVA light remained stable (Figure S7B). Transient laser absorbance spectroscopy was used to inspect potential reactivity with the triplet state of perinaphthenone and potential kinetic solvent isotope effects on triplet reactivity. No significant reactivity of polymyxin with triplet perinaphthenone was detected (Figure S8 and Table S9).
A kinetic analysis of polymyxin transformation products further supports that oxidation indeed took place. Data in Figure 3B show the kinetic traces of the main transformation products observed for polymyxins E2 with m/z mass shifts relative to the parent of [+66], [+96], [+132], [+162] and [+192] (Tables S10-S11, extracted ion chromatograms, full-scan MS1 spectra Figures S9-S10). Oxidative modifications with mass addition of +66 m/z and +96 m/z, among other minor products, were observed for all polymyxin variants (B1, B2, E1, E2). These common products indicate that the oxidation occurred at moieties that are retained in all four polymyxin variants, i.e., not at the variable phenylalanine/leucine moiety nor the modified alkyl chain.

Figure 3. Phototransformation in sunlight and product formation by reaction with singlet oxygen. (A) Degradation in simulated sunlight in the presence of dissolved organic matter, (Suwannee River Fulvic Acid, 10 mg L\(^{-1}\)) for daptomycin (red triangles), bacitracins A1, B1, and C1 (superimposed blue circles), polymyxins B1 and B2 (superimposed green squares) and polymyxins E1 & E2 (superimposed purple diamonds). The singlet oxygen steady state concentration \([1^O_2]_{ss}\) ranged from 1.8 to 3.8 x 10\(^{-13}\) M in the DOM containing samples. (B) Kinetic traces of relative peaks areas for the main phototransformation products observed for the reaction of polymyxin E2 with singlet oxygen (UVA exposure with sensitizer perinaphthenone at 0.7 µM). Mass shifts relative to the parent ion are indicated in square brackets. Error bars represent one standard deviation (n=3).

The 2,4-diaminobutyric acid (Dab) is present in five positions in polymyxins, twice in the linear side chain and three times in the cyclic part, with one Dab building the ring closure by forming an ureido bond connecting the primary amine of the Dab R-group with the C-terminal end of threonine Figure ). Diagnostic evidence of the MS\(^2\) fragmentation of the [+66] products suggest that these mass additions can be found on fragments that always contained at least one Dab moiety. The Dab ion alone has a m/z of 101.0712 and fragments of m/z 167.0712 were observed, equalling a mass shift of +66 m/z. Despite the evidence for a reaction at one or more Dab moieties, the chemical structure does not intuitively suggest a reaction with \(^1O_2\) Dab is similar in structure to the amino acids lysine and ornithine, with a modification in the aliphatic side chain (Figure S11). Canonical amino acids have been studied extensively regarding their photoreactivity and no report exists to our knowledge that demonstrates that lysine is reactive towards \(^1O_2\). The bacitracins and daptomycin all contain an ornithine residue,
but until now, there has been no clear evidence that these moieties are susceptible to oxidation by $^{1}\text{O}_2$. The reactivity of Dab with discrete oxidants in free solution and in a simple combined tripeptide should be tested to further elucidate whether the reactivity of polymyxins can be attributed to this monomer.

4.3.3 Biotransformation potential by periphyton Lastly, we investigated the potential of biologically mediated transformation of the AMPs by a periphyton community in concentrated riverine biofilm suspensions. The biological activity of the biofilm suspensions was verified by co-spiking benzoic acid as a readily degradable substrate. Benzoic acid was consumed below the detection limit within the first 24 hours, but was stable in all samples that underwent autoclaving and in the presence of AMPs in ultrapure water. (Figure S12). Data in Figure 4A-B show that concentrations of daptomycin and bacitracin A decreased with pseudo-first-order kinetics from the aqueous phase in the presence of active biofilm suspensions by more than 95% for daptomycin, 80-82% for bacitracin A1, and 86-90% for bacitracins B1 and C1 within 4 days (Figure 4, green filled diamonds, solid lines). Comparable kinetics were observed for bacitracin B1 and C1 (Figure S13). Daptomycin remained stable over 4 days exposure to the biofilm suspension that was inactivated by repeated autoclaving (Figure 4A, green open circles, dash-dotted line). The observed removal in these batch exposure tests was reproducible for active biofilm suspensions from two different cultures (June and August 2018) and comparable results were obtained with sacrificial exposure tests. However, one cycle of autoclaving did not inactivate the biofilm suspension sufficiently, which was also apparent for bacitracin (Figure 4B, green open diamonds, dashed lines). The initial kinetics suggest that sorption to particulates in suspension was not dominating the removal process for daptomycin and bacitracins. Analogous to the sorption kinetic experiments with terrestrial soils, a 30-hour control experiment was conducted with a higher sampling frequency with biofilm suspension that was inactivated (autoclaving and additional freeze-thaw cycle) and filtered biofilm suspension (particle-free). All bacitracin variants and daptomycin concentrations remained stable indicating that neither sorption to particulate biofilm material nor transformation in the particle-free supernatant took place (Figure 4, open green squares and orange triangles, respectively). For polymyxins, the concentration of all variants decreased below the limit of quantification in the supernatant after 24 hours of exposure to active biofilm suspensions, similar to benzoic acid. However, in the autoclaved controls, removal was only observed within the first 24 hours and all concentrations remained constant and above the limit of quantification for the subsequent days (Figure S14). The initial decrease of up to 86% after 9 hours can be attributed to sorption to the particulate matter. The fast additional loss in the active sample is likely mediated by the active periphyton community. We conclude that active riverine biofilm communities are capable of successfully removing these AMPs from the aqueous phase. The removal from concentrated biofilm suspensions encourages future work with long-term exposure to natural waters to assess transformation rates at environmental conditions.
4.3.3.1. **Biotransformation products.** One major transformation product of daptomycin was observed, \( m/z = 818.8589 \), that appeared upon exposure to an active biofilm suspension and reached a maximum intensity after 1 day, before it started to gradually disappear (Figure 4C). This product was not observed in the autoclaved samples. This daptomycin transformation product has previously been reported upon exposure to environmental actinomycetes and wastewater microbial communities and can result from enzymatic hydroxylation of an aliphatic carbon \( (m/z \text{ shift of } [+16])^{161,162} \). While the presented experiments were not designed to specifically follow transformation products, (i.e., low spike concentrations), the appearance of this transformation product further supports that biologically mediated transformation occurred for daptomycin. For bacitracins, previous literature demonstrated microbial transformation of bacitracin A by enzymatic hydrolysis of the ester or peptide bonds by the bacterial strain B-9 isolated from a lake in Japan.\(^{163} \) These transformation products were reported to be non-stable, which may be the reason why we did not identify them in our samples by suspect screening.

![Figure 4](image-url)

**Figure 4. AMP removal during exposure to biofilm suspensions** for (A) Daptomycin (0.2 µM) and (B) Bacitracin A (2 µM) in 40 mL batch test for active biofilm suspension (green, filled diamonds), 1-time autoclaved suspensions (green, open diamonds) and 2-times autoclaved suspensions (green, open circles), and a kinetic sorption test for up to 30 hours with biofilm suspension that was 1-time autoclaved and underwent an additional freeze-thaw cycle for inactivation unfiltered (green, open squares) and filtered (0.45 µm), (orange, open triangles). (C) Daptomycin product formation with \( m/z = 818.8589 \) as relative peak areas of the extracted ion chromatogram and error bars represent the standard deviation of 3-6 replicates.

4.4 Conclusions

The presented work contributes to a better understanding of the environmental behaviour of AMPs. To our current knowledge, we present the first empirical values for soil-water partitioning coefficients for daptomycin and bacitracins. In the absence of empirical data, models used to predict environmental partitioning mostly rely on calculated octanol-water partitioning coefficients \( (K_{ow} \text{ values}) \), which range over up to 10 log units for these AMPs. Our data demonstrates that sorption to soils and organic material plays an important role in the environmental fate of AMPs but can depend strongly on the soil type and speciation of AMP moieties. Current models to determine predicted environmental concentrations of AMPs do not consider environmental transformation processes because the
required data is not available. Our study suggests that all AMPs tested are labile to photochemical transformation processes and we report bimolecular transformation rate constants with singlet oxygen that can readily be applied in modelling efforts. All AMPs also showed biotransformation potential by periphyton biofilm communities, which encourages future work to identify biotic transformation pathways.

Acknowledgements
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4.5 References


Chapter S4. Supplementary Information: Fate Processes of Antimicrobial Peptides Daptomycin, Bacitracin, and Polymyxins in Environmental Systems

The electronic supplementary information contains 14 figures and 11 tables and 3 texts with experimental details about the calculation of concentrations for individual antimicrobial peptide variants, calculation of new m/z values upon H/D exchange and the derivatization of polymyxins with the fluorescence tag 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC).
Table S1. Characterization of important soil parameters for Standard LUFA soils 2.1 and 6S.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Soil Organic C (%)</th>
<th>Soil Organic N (%)</th>
<th>Cation Exchange Capacity (meq/100 g)</th>
<th>Maximum Water Holding Capacity (g/100 g)</th>
<th>Density (g/1000 ml)</th>
<th>Clay (%) (particle size &lt;0.002 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>0.71 ± 0.07</td>
<td>0.06 ± 0.01</td>
<td>4.2 ± 0.6</td>
<td>32.5 ± 1.6</td>
<td>1436 ± 41</td>
<td>2.8 ± 0.07</td>
</tr>
<tr>
<td>6S</td>
<td>1.77 ± 0.08</td>
<td>0.18 ± 0.01</td>
<td>26.5 ± 2.6</td>
<td>40.8 ± 1.4</td>
<td>1352 ± 42</td>
<td>41.7 ± 1.1</td>
</tr>
</tbody>
</table>

Table S2. Values of pH of 10 mM CaCl₂ supernatant after 48 h equilibration with different soils on horizontal shaker.

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUFA 2.1 - 1</td>
<td>4.69</td>
</tr>
<tr>
<td>LUFA 2.1 - 2</td>
<td>4.71</td>
</tr>
<tr>
<td>LUFA 2.1 - average</td>
<td>4.70</td>
</tr>
<tr>
<td>LUFA 6S - 1</td>
<td>6.90</td>
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<tr>
<td>LUFA 6S - 2</td>
<td>6.86</td>
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<tr>
<td>LUFA 6S - average</td>
<td>6.88</td>
</tr>
</tbody>
</table>

Table S3. Weight of 50 mg soil before and after drying overnight at 105°C.

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>vial + soil before heating (g)</th>
<th>vial + soil after heating (g)</th>
<th>dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUFA 2.1 - 1</td>
<td>2.4761</td>
<td>2.4756</td>
<td>0.0495</td>
</tr>
<tr>
<td>LUFA 2.1 - 2</td>
<td>2.4604</td>
<td>2.4604</td>
<td>0.0500</td>
</tr>
<tr>
<td>LUFA 2.1 - 3</td>
<td>2.5154</td>
<td>2.5150</td>
<td>0.0496</td>
</tr>
<tr>
<td>LUFA 2.1 - average</td>
<td></td>
<td></td>
<td>0.0497</td>
</tr>
<tr>
<td>LUFA 6S - 1</td>
<td>2.5097</td>
<td>2.5086</td>
<td>0.0489</td>
</tr>
<tr>
<td>LUFA 6S - 2</td>
<td>2.5055</td>
<td>2.5046</td>
<td>0.0491</td>
</tr>
<tr>
<td>LUFA 6S - 3</td>
<td>2.4720</td>
<td>2.4744</td>
<td>0.0491</td>
</tr>
<tr>
<td>LUFA 6S - average</td>
<td></td>
<td></td>
<td>0.0490</td>
</tr>
</tbody>
</table>
Figure S1 Concentration vs. time for selected AMPs incubated with soil and soil supernatant (10 mM CaCl₂) for up to 30 hours. Polymyxin E1 and E2 were no longer detected in the supernatant after coming in contact with soils.

“Supernatant” samples: 10 mM CaCl₂ was incubated with soil overnight (~12 h), then the soil was removed using centrifugation (10000xg using the microcentrifuge). This supernatant was transferred into a 1 mL HPLC vial, and AMPs were spiked. This media was also used to prepare the “matrix-matched” calibration curves.
Table S4. Measured pH values and ash-free dry weight for biofilm slurry.

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>pH</th>
<th>Dry weight* (mg)</th>
<th>Ash-free Dry Weight** (mg) / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>active - 1</td>
<td>7.97</td>
<td>4.42</td>
<td>3.0 / 68%</td>
</tr>
<tr>
<td>active - 2</td>
<td>8.03</td>
<td>4.45</td>
<td>3.3 / 74%</td>
</tr>
<tr>
<td>active - 3</td>
<td>8.19</td>
<td>5.11</td>
<td>3.5 / 69%</td>
</tr>
<tr>
<td>active (average)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>autoclaved - 1</td>
<td>7.73</td>
<td>4.63</td>
<td>3.1 / 67%</td>
</tr>
<tr>
<td>autoclaved - 2</td>
<td>7.78</td>
<td>5.16</td>
<td>3.4 / 66%</td>
</tr>
<tr>
<td>autoclaved - 3</td>
<td>7.71</td>
<td>5.63</td>
<td>3.3 / 59%</td>
</tr>
<tr>
<td>autoclaved – (average)</td>
<td></td>
<td>5.14</td>
<td>3.3 / 64%</td>
</tr>
</tbody>
</table>

*dry weights were calculated from the weight of biofilm material retained from 10 mL of biofilm slurry on a GF/F glass microfiber filter (0.7 µm) after drying in an oven at 105°C for 1 hour.

**ash-free dry weights were calculated from the weight that was lost when combusting the biofilm material retained on a GF/F glass microfiber filter (0.7 µm) at 480°C for 1 hour.

Table S5. HPLC gradient for measuring AMPs on a Poroshell 120 EC-C18 column.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>6.1</td>
<td>90</td>
</tr>
<tr>
<td>9</td>
<td>90</td>
</tr>
<tr>
<td>9.1</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>10 (end of method)</td>
</tr>
</tbody>
</table>

Figure S2 Comparison of calibration curves for Bacitracin A1 in various matrices. Grey filled circles display filtered (0.2 µm) biofilm, orange filled squares shows soil supernatant of LUFA 2.1, blue filled triangles represent soil supernatant of LUFA 6S, and red filled diamonds correspond to samples in nanopure H₂O.
Text S1. Calculation of concentrations for AMP mixture components.

The polymyxins and bacitracin variants are sold commercially as mixtures: Polymyxin B1 and B2 are together, as well as Polymyxin E1 and E2 (also referred to as Colistin A and B) and both are in the form of the sulphate salt. Bacitracin A, B, and C are also sold as one formula, as a zinc salt. The relative concentrations were calculated assuming that the ionization efficiency was the same for each variant. The ratio of variants for each point of the calibration curve was calculated, and then averaged: Polymyxin B1: 43%, Polymyxin B2: 57%, Polymyxin E1: 37%, Polymyxin E2: 63%, Bacitracin A: 52%, Bacitracin B: 38%, Bacitracin C: 10%. Actual stock solution concentrations were: Polymyxin B mixture: 1040 mg/L, Polymyxin E mixture: 906 mg/L, Bacitracin mixture: 1140 mg/L.

Table S6. Suspect list for biotransformation products as reported in the literature.

<table>
<thead>
<tr>
<th>Parent AMP</th>
<th>m/z of product</th>
<th>Type of Biotransformation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacitracin A1</td>
<td>721 (720.8674)*</td>
<td>Hydrolysis of His-Phe peptide bond</td>
<td>163</td>
</tr>
<tr>
<td>Bacitracin A1</td>
<td>647 (647.2439)*</td>
<td>Cleavage at Ile-Phe peptide bond</td>
<td>163</td>
</tr>
<tr>
<td>Bacitracin A1</td>
<td>521 (521.8085)*</td>
<td>Hydrolysis of His-Asp peptide bond</td>
<td>163</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>1639 (819.3638)*</td>
<td>Ring hydrolysis, likely at the ester bond</td>
<td>161</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>1467 (732.2906)*</td>
<td>Deacylation of lipid tail</td>
<td>161</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>1485 (742.7998)*</td>
<td>Cleavage at ester bond and acyl sidechain</td>
<td>161</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>1299 (649.7602)*</td>
<td>Cleavage at ester bond and peptide bond (before Trp)</td>
<td>161</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>818.8599</td>
<td>Hydroxylation of an aliphatic carbon</td>
<td>162</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>733.7946</td>
<td>Hydrolysis of secondary amide</td>
<td>162</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>640.7549</td>
<td>Hydrolysis of secondary amide</td>
<td>162</td>
</tr>
</tbody>
</table>

*accurate m/z calculated for [M+2H]²⁺ with enviPat Web 2.4 (www.envipat.eawag.ch) from structures drawn in ChemDraw Professional (16.0)

References:

Text S2. Calculation of changes in the m/z upon H/D exchange of amino protons.

The number of amino, or “H/D exchangeable” protons was determined for daptomycin (26), and polymyxins B1, B2, E1 and E2 (28). An R-script was created to predict new m/z values for all isotopes, based on 0, 25%, 50%, 75% and 100% exchange of D for H into their respective molecular formulas. The sum of these new possible masses at the retention time of the AMP of interest was used to quantify the deuterated parent compound.

Text S3. AQC derivatization method for fluorescence tagging of polymyxins.

On-line derivatization with autosampler: Autosampler mixed 4 µL sample with 8 µL borate buffer (100 mM) and 2 µL of 5.1 mM AQC solution in dry acetonitrile (reagent very sensitive to reactions with O₂ and H₂O, therefore LC vials were filled under an argon atmosphere and crimped shut). Tagged polymyxins were measured using HPLC-FLD detection (excitation λ = 245, emission λ = 395 nm). The aqueous eluent (A) consisted of an acetate buffer (pH 5.9) and organic eluent (B) was 100% acetonitrile. A gradient method was used, 0-2 min: 10% B, 2.1 min: 40% B, 13 min: 46% B, 14-18 min: 90% B, 8.1-20 min: 10% B.

Figure S3 Comparison of pseudo-first order degradation kinetics of polymyxin E under UVA irradiation in the presence of 1 µM perinaphthenone in H₂O (black hollow circles), and 90% D₂O (blue triangles). Red squares show direct controls in H₂O, Panel A shows polymyxin E with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization (fluorescence detection) and panel B shows Polymyxin E with LC-MS detection.
Table S7. Calculated $K_{ow}$ values in literature from model prediction software.

<table>
<thead>
<tr>
<th>AMP</th>
<th>Pubchem</th>
<th>ChemAxon</th>
<th>ALOGPS</th>
<th>EPISuite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacitracin A</td>
<td>-0.8</td>
<td>-7.25</td>
<td>-2.97</td>
<td>-3.26</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>-5.1</td>
<td>-9.37</td>
<td>-0.47</td>
<td>-9.77</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>-2.5</td>
<td>-7.2</td>
<td>-0.89</td>
<td>-6.04</td>
</tr>
<tr>
<td>Polymyxin E</td>
<td>-3.3</td>
<td>0.92</td>
<td>0.22</td>
<td>-6.83</td>
</tr>
</tbody>
</table>

Figure S4. UV-vis spectra for AMPs and spectral output of the solar simulator

Figure S5. Comparison of pseudo-first order degradation kinetics of (A) daptomycin and (B) polymyxin B1 & B2 (superimposed) under UVA irradiation in the presence of 1 µM perinaphthenone in H$_2$O (black hollow circles), and 90% D$_2$O (blue triangles).
Table S8. List of photooxidation products observed for Daptomycin under UVA irradiation in the presence of perinaphthenone in H₂O.

<table>
<thead>
<tr>
<th>Transformation Product ID</th>
<th>z=2</th>
<th>z=1</th>
<th>Mass shift from Parent (Da)</th>
<th>composition</th>
<th>Suggested Formula [M+H]⁺</th>
<th>Delta (ppm)</th>
<th>Supposed Oxidation Pathways*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP-826</td>
<td>826.8526</td>
<td>1652.7015</td>
<td>31.984</td>
<td>2(O)</td>
<td>C72H101O28N17</td>
<td>-3.613</td>
<td><strong>Trp</strong> → <strong>Trp-OH [+16]</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>Kyn</strong> → <strong>Kyn-OH [+16]</strong></td>
</tr>
<tr>
<td>TP-834</td>
<td>834.8480</td>
<td>1666.6790</td>
<td>45.961</td>
<td>3(O)</td>
<td>C72H101O29N17</td>
<td>-4.642</td>
<td><strong>Trp</strong> → <strong>Trp-dione [+30]</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>Kyn</strong> → <strong>Kyn-OH [+16]</strong></td>
</tr>
<tr>
<td>TP-817</td>
<td>817.8497</td>
<td>1634.6989</td>
<td>13.981</td>
<td>1(O) – 2H</td>
<td>C72H99O27N17</td>
<td>-2.56</td>
<td></td>
</tr>
<tr>
<td>TP-825</td>
<td>825.8470</td>
<td>1650.6902</td>
<td>29.973</td>
<td>2(O) – 2H</td>
<td>C72H99O28N17</td>
<td>2.576</td>
<td><strong>Trp</strong> → <strong>Trp-dione [+30]</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>Or [+14] and Kyn</strong> → <strong>Kyn-OH [+16]</strong></td>
</tr>
<tr>
<td>TP-841</td>
<td>841.8393</td>
<td>1680.6644</td>
<td>59.947</td>
<td>4(O) – 2H</td>
<td>C72H99O30N17</td>
<td>-0.952</td>
<td><strong>Trp</strong> → <strong>OH-bis Trp-dione [+44]</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>Kyn</strong> → <strong>Kyn-OH [+16]</strong></td>
</tr>
<tr>
<td>TP-850</td>
<td>850.8451</td>
<td>1700.6855</td>
<td>79.968</td>
<td>5(O)</td>
<td>C72H101O31N17</td>
<td>-3.949</td>
<td><strong>Trp</strong> → <strong>diOH-NFK [+64]</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>Kyn</strong> → <strong>Kyn-OH [+16]</strong></td>
</tr>
</tbody>
</table>

*based on reported tryptophan and kynurenine oxidation products (Nakagawa et al. 1977, Davies et al. 2001, Plowman et al. 2013)

TP = transformation product; Trp = tryptophan; Kyn = kynurenine; NFK = n-formyl kynurenine
Figure S6. Kinetic plots of daptomycin oxidation products during exposure to UVA light and in the presence of 1 µM perinaphthenone. The product [+14] was observed but could not be assigned to a known transformation product of tryptophan.

Figure S7. KIE on pseudo-first order degradation kinetic of AMPs for “direct phototransformation” (no sensitizer added) in H$_2$O (black hollow circles) and in D$_2$O (red filled squares). Panel A shows Daptomycin and Panel B shows Polymyxin B1 and B2 variants together. No reaction rate constants could be calculated for the Polymyxin data due to large error between replicate data points (n=3).
Figure S8. Transient absorption spectroscopy of 100 µM perinaphthenone, in various media (100% H₂O or 100% D₂O), in the presence and absence of quencher molecules. ³PN* signal was averaged from 475 nm – 495 nm (average shown above). 300 µM polymyxin B or E did not appear to quench the lifetime of ³PN* in either media. Calculated lifetimes shown in Table A9 below.

Table S9. Transient absorption spectroscopy of 100 µM perinaphthenone, in various media (100% H₂O or 100% D₂O), in the presence and absence of quencher molecules.

<table>
<thead>
<tr>
<th>Triplet Quencher</th>
<th>³PN* lifetime ± error (µs) 100% H₂O</th>
<th>³PN* lifetime ± error (µs) 100% D₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>No quencher (native lifetime)</td>
<td>6.42 ± 0.11</td>
<td>6.62 ± 0.07</td>
</tr>
<tr>
<td>300 µM PMB</td>
<td>6.42 ± 0.15</td>
<td>6.89 ± 0.16</td>
</tr>
<tr>
<td>300 µM PME</td>
<td>6.39 ± 0.15</td>
<td>6.76 ± 0.17</td>
</tr>
</tbody>
</table>

The ³PN* lifetimes were slightly longer in D₂O, versus H₂O (3-7%), however, high concentrations of the drugs did not quench the triplet lifetimes, and therefore ³PN*, was not found to be reactive with polymyxins.
### Table S10. List of photooxidation products observed for Polymyxin B under UVA irradiation in the presence of perinaphthenone in H₂O.

<table>
<thead>
<tr>
<th>Transformation Product ID</th>
<th>Parent Compound</th>
<th>z=2</th>
<th>z=1</th>
<th>Mass shift from Parent (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP-628</td>
<td>Polymyxin B2</td>
<td>628.3781</td>
<td>1255.7499</td>
<td>+ 66.008</td>
</tr>
<tr>
<td>TP-635</td>
<td>Polymyxin B1</td>
<td>635.3857</td>
<td>1269.7652</td>
<td>+ 66.008</td>
</tr>
<tr>
<td>TP-638</td>
<td>Polymyxin B2</td>
<td>638.3726</td>
<td>1277.7316</td>
<td>+ 87.990</td>
</tr>
<tr>
<td>TP-646</td>
<td>Polymyxin B1</td>
<td>646.3773</td>
<td>1291.7470</td>
<td>+ 87.990</td>
</tr>
<tr>
<td>TP-643</td>
<td>Polymyxin B2</td>
<td>643.3832</td>
<td>1285.7602</td>
<td>+ 96.019</td>
</tr>
<tr>
<td>TP-650</td>
<td>Polymyxin B1</td>
<td>650.3909</td>
<td>1299.7758</td>
<td>+ 96.019</td>
</tr>
<tr>
<td>TP-661</td>
<td>Polymyxin B2</td>
<td>661.383</td>
<td>1321.7599</td>
<td>+ 132.018</td>
</tr>
<tr>
<td>TP-668</td>
<td>Polymyxin B1</td>
<td>668.3912</td>
<td>1335.7758</td>
<td>+ 132.018</td>
</tr>
<tr>
<td>TP-676</td>
<td>Polymyxin B2</td>
<td>676.3883</td>
<td>1351.7704</td>
<td>+ 162.029</td>
</tr>
<tr>
<td>TP-683</td>
<td>Polymyxin B1</td>
<td>683.3961</td>
<td>1365.7865</td>
<td>+ 162.029</td>
</tr>
<tr>
<td>TP-691</td>
<td>Polymyxin B2</td>
<td>691.3937</td>
<td>1381.7813</td>
<td>+ 192.040</td>
</tr>
<tr>
<td>TP-698</td>
<td>Polymyxin B1</td>
<td>698.4013</td>
<td>1395.7973</td>
<td>+ 192.040</td>
</tr>
</tbody>
</table>

### Table S11. List of photooxidation products observed for Polymyxin E under UVA irradiation in the presence of perinaphthenone in H₂O.

<table>
<thead>
<tr>
<th>Transformation Product ID</th>
<th>Parent Compound</th>
<th>z=2</th>
<th>z=1</th>
<th>Mass shift from Parent (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP-611</td>
<td>Polymyxin E2</td>
<td>611.3865</td>
<td>1221.7655</td>
<td>+ 66.008</td>
</tr>
<tr>
<td>TP-618</td>
<td>Polymyxin E1</td>
<td>618.3811</td>
<td>1235.7809</td>
<td>+ 66.008</td>
</tr>
<tr>
<td>TP-622</td>
<td>Polymyxin E2</td>
<td>622.3770</td>
<td>1243.7472</td>
<td>+ 87.990</td>
</tr>
<tr>
<td>TP-629</td>
<td>Polymyxin E1</td>
<td>629.3803</td>
<td>1257.7627</td>
<td>+ 87.990</td>
</tr>
<tr>
<td>TP-626</td>
<td>Polymyxin E2</td>
<td>626.3915</td>
<td>1251.7758</td>
<td>+ 96.019</td>
</tr>
<tr>
<td>TP-633</td>
<td>Polymyxin E1</td>
<td>633.3990</td>
<td>1265.7915</td>
<td>+ 96.019</td>
</tr>
<tr>
<td>TP-644</td>
<td>Polymyxin E2</td>
<td>644.3914</td>
<td>1287.7755</td>
<td>+ 132.018</td>
</tr>
<tr>
<td>TP-651</td>
<td>Polymyxin E1</td>
<td>651.3995</td>
<td>1301.7915</td>
<td>+ 132.018</td>
</tr>
<tr>
<td>TP-659</td>
<td>Polymyxin E2</td>
<td>659.3967</td>
<td>1317.7860</td>
<td>+ 162.029</td>
</tr>
<tr>
<td>TP-666</td>
<td>Polymyxin E1</td>
<td>666.4046</td>
<td>1331.8022</td>
<td>+ 162.029</td>
</tr>
<tr>
<td>TP-674</td>
<td>Polymyxin E2</td>
<td>674.4020</td>
<td>1347.7969</td>
<td>+ 192.040</td>
</tr>
<tr>
<td>TP-681</td>
<td>Polymyxin E1</td>
<td>681.4100</td>
<td>1361.8130</td>
<td>+ 192.040</td>
</tr>
</tbody>
</table>
Figure S9. Extracted ion chromatograms for new polymyxin E2 transformation product peaks.

Figure S10. Total ion chromatogram for the full-scan MS1. The same modifications are observed for polymyxin E1 (PME1) and polymyxin E2 (PME2).
Figure S11. Chemical structures for structurally similar primary amine amino acids 2,4-diaminobutyric acid, ornithine, and lysine.
Chapter Five: Conclusions and Outlook

**Figure S12.** Benzoic acid removal during exposure to biofilm suspensions and ultrapure water. 10 µM benzoic acid co-spiked with daptomycin (blue diamonds), polymyxin B (1&2) (red squares), bacitracin (A; B, C) (green triangles), and polymyxin E (1&2) (orange circles). Benzoic acid could no longer be detected in active biofilm samples after day 0. Error bars represent the standard deviation of 3-6 replicates.

**Figure S13.** Bacitracin B and C removal during exposure to biofilm suspensions. (A) bacitracin B (1.34 mg/L), and (B) bacitracin C (0.27 mg/L) in 40 mL batch test for active biofilm suspension (green, filled diamonds), 1-time autoclaved suspensions (green, open diamonds). Error bars represent the standard deviation of 3-6 replicates.
Figure S14. Polymyxin removal during exposure to biofilm suspensions and ultrapure water (MQ). (A) polymyxin E1, (B) polymyxin E2, (C) polymyxin B1, and (D) polymyxin B2 in 40 mL batch test for 1-time autoclaved suspensions (green, open diamonds), and ultrapure water (blue, hollow squares). Polymyxin could not be detected in active biofilm suspensions after 0 days. Data points from replicate samples (3) are superimposed.
Chapter 5. Conclusions and Outlook

This dissertation provided a detailed investigation of the photochemical pathways for two groups of environmentally relevant pharmaceuticals. Special attention was dedicated to determining the role of singlet oxygen in indirect photochemistry, and the experimental methods available for photochemists to do so.

As a result of Chapter 2, direct phototransformation was found to be significant for most of the diarylamines (aside from mefenamic acid) in the top layer of a water body. Indirect phototransformation became more important further down the photic zone of the water column for all diarylamines. Bimolecular reaction rate constants for environmentally relevant PPRIs (•OH, ¹O₂) and a model triplet sensitizer (³sens*) were determined, as well as steady-state concentrations of these PPRIs generated by DOM under simulated sunlight conditions. The dual roles of DOM as a sensitizer and antioxidant toward these pharmaceuticals were demonstrated by transient absorption spectroscopy. On the one hand, these tests confirmed that triplet excited states of triplet sensitizers can oxidize diarylamines by an electron abstraction as the formation of diarylamine-based radical intermediates were observed. On the other hand, these diarylamine-based radical intermediates could be quenched by a model antioxidant and bimolecular reaction rate constants could be determined.

The findings in Chapter 3 demonstrated a novel non-singlet oxygen related kinetic solvent isotope effect in aquatic photochemistry. Specifically, the reduction of diarylamine-based radical intermediates by antioxidants to their parent compound also shows a kinetic solvent isotope effect. The effect was demonstrated by transient laser spectroscopy, where the radical intermediates were quenched slower in D₂O compared to H₂O. Consequently, one must be mindful when relying on the kinetic solvent isotope effect to determine the reactivity specifically with ¹O₂, especially when reductants like DOM are present in reaction mixtures. Conversely, differences in kinetic solvent isotope effects in the presence and absence of antioxidants could be used as diagnostic evidence of a reversible electron transfer oxidation mechanism. Data suggests that neither the reactivity of triplets towards organic compounds, nor the triplet lifetimes were affected by the change in solvents.

The results from Chapter 4 provided the first insights into the environmental behaviour of antimicrobial peptides daptomycin, bacitracins, and polymyxins. We found that sorption can play a dominating role and determined the soil-water partitioning coefficients. These empirical values will provide more accurate parameters for models assessing environmental concentration of antimicrobial peptides compared to current predictions that rely on in-silico octanol-water partitioning coefficients, which can vary in magnitude by up to 10 log units. In addition, the antimicrobial peptides can all undergo indirect phototransformation reaction, especially with ¹O₂ in surface waters. The particularly high reactivity with
polymyxins was not expected based on known reaction mechanisms of $^1$O$_2$. Finally, all antimicrobial peptides exhibited potential to be biotransformed by riverine-derived biofilm.

**Outlook**

As scientists, we are perpetually curious, and as soon as some questions are answered, we are presented with a wealth of new research avenues and areas to further improve our understanding. Based on the results presented in this dissertation, several opportunities as areas for future studies can be identified:

(i) probing what drives the singlet oxygen reactivity with polymyxins
(ii) evaluating potential photochemical reactions of pharmaceuticals sorbed to organic or mineral particles
(iii) evaluating the activity of pharmaceuticals after sorption and bio-/photo-transformation.

5.1 Probing what drives singlet oxygen reactivity with polymyxins

As stated in chapter 4, polymyxins reacted with high bimolecular rate constants with $^1$O$_2$. This result was relatively surprising considering that none of polymyxins’ amino acid building blocks are known to react with $^1$O$_2$. Based on initial analysis of the oxidized transformation products, and their MS$^2$ fragmentation, one can hypothesize that the modifications occurred one of the 2,4-diaminobutyric acid (Dab) moieties. On the other hand, no reactivity has been reported for structurally related molecules ornithine, and lysine. The specific reactivity of 2,4-diaminobutyric acid with $^1$O$_2$ should be evaluated while freely dissolved in solution, as well as in combined peptides with matching neighboring amino acids as are found in polymyxin (i.e., Dab-Thr-Dab, Thr-Dab-Dab, Dab-Dab-Leu, Dab-Dab-Dab).

Furthermore, the potential reactivity with triplet excited state sensitizers also present in these $^1$O$_2$ experiments and reaction with hydroxy radicals should be evaluated.

In addition, the reactivity of $^1$O$_2$ with polymyxins can be further evaluated by improving the analysis of transformation products by MS$^3$ mass spectrometry. More strategic MS fragmentation can yield site-specific elucidation of the structure of transformation products, including the positions of the modifications. In the current data set transformation product signals were high enough to trigger MS$^2$ measurements automatically in a top 5 data dependent MS$^2$ run, beyond the parent ions in the inclusion list. These $m/z$ values for the main transformation products should be extended and used as an inclusion list for tandem-mass spectrometry experiments in the future. The peptides should also be fragmented with different collision energies and by MS$^n$ to generate a larger variety of fragments. In combination with improved fragmentation for diagnostic evidence of the transformation products, an annotation software can assist to interpret fragmentation of these complex cyclic peptides. One example is CycloBranch, a free, open-source software that uses de novo sequencing for the
identification and detailed characterization of nonribosomal peptides and has been validated with a number of linear, cyclic, branched, and cyclic branched peptides.\textsuperscript{164}

5.2 Evaluating Potential Abiotic Transformation of Pharmaceuticals Sorbed to Organic or Mineral Particles

Sorption was an influential process in the environmental fate of antimicrobial peptides, yet it is not known whether further transformation processes can also occur for peptides sorbed to organic and mineral particles. Previous evidence suggests that photochemical processes can occur also on particle surfaces for sorbed molecules. In the late 1970s, Zepp and co-workers demonstrated that suspended sediments could influence the photolysis rates of dissolved pollutants.\textsuperscript{165} They found that photolysis rates in the photic zone of turbid water were generally faster in turbid compared to clear water, after accounting for light attenuation by the sediment. They attributed enhanced photolysis rates to increased diffuseness of light due to scattering. The same group also revealed that photoreactivity was affected for aquatic pollutants sorbed on suspended sediments.\textsuperscript{166} The main findings showed that the photoreactivity for hydrophobic chemicals was increased because sorbed chemicals were in a less polar microenvironment, which serves as a considerably better hydrogen donor than water. They stated that the differences in photochemical behaviour of sorbed cationic and nonpolar chemicals depends highly on the sorption site. Generally, cations adsorb primarily to the clay mineral parts of sediments, whereas nonpolar chemicals associate with the organic part.

A more recent study compared at the phototransformation kinetics of the freely dissolved antibiotic tetracycline to reaction kinetics of the drug adsorbed onto two different model smectite clays (hectorite and montmorillonite).\textsuperscript{167} They observed that adsorption on clays accelerated the phototransformation of tetracycline and attributed this enhancement to the facilitated production of self-sensitized singlet oxygen. Another study of sorption and phototransformation, this time of the antibiotics tylosin and sulfamethazine, used humic acid-coated goethite as a sorbent material.\textsuperscript{168} They observed that the phototransformation of the two compounds increased with increasing humic acid concentration, which they attributed to increased concentrations of Fe(II) and H$_2$O$_2$. Besides phototransformation, abiotic hydrolysis and oxidation of peptide bonds has been recently reported for proteins associated with mineral surfaces.\textsuperscript{169}

These studies strongly investigating transformation processes of organic molecules on surfaces, especially for substances that have been proven to sorb to soil particles.

5.3 Evaluating the Activity of Pharmaceuticals After Sorption and Bio-/Photo-transformation

Pharmaceuticals contribute critically to environmental pollution because they are designed to be biologically active, which can impact non-target organisms in the ecosystem. Especially the distribution of antimicrobial drugs contributes to the spread of antibiotic resistance if non-lethal
concentrations of the drugs are omnipresent in the environment, potentially spurring a selective pressure on bacteria. The work performed for this dissertation has shown that the investigated pharmaceuticals were able to transform or leave the aqueous phase via different mechanisms. An important follow-up question would be whether the transformation products, or sorbed forms of the pharmaceuticals still have their designed pharmaceutical mode of action or cause other ecotoxicological effects. To determine this experimentally, activity assays could be performed using the final reaction mixture from phototransformation, biotransformation, and suspended particles of sorption experiments. The activity of the diarylamine nonsteroidal anti-inflammatory drugs, can be tested with a COX-2 (human) Inhibitor Screening Assay Kits, which are commercially available. For the antimicrobial peptides, minimum inhibitory concentration assays (MIC) can be performed for a target bacterium (e.g., gram negative *Pseudomonas aeruginosa* for polymyxins, gram positive *Staphylococcus aureus* for daptomycin and bacitracin). Such MIC assays were conducted successfully in preliminary experiments in context of this dissertation (data not shown).
5.4 References


Curriculum Vitae

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## Education

<table>
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<tr>
<th>Institution</th>
<th>Degree</th>
<th>Field</th>
<th>Advised by</th>
<th>Duration</th>
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| **ETH Zurich**  
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Department of Environmental Systems Science | Dr. Elisabeth Janssen & Prof. Kristopher McNeill | 2015-2019 |
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## Research Experience

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<tr>
<th>Institution</th>
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| **ETH Zurich / Eawag**  
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