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 promotors 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 bi-molecular 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.
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 (Butaye et al., 2003; Katsumuma et al., 2007; Phillips, 1999; Rhouma et al., 2016). 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 bacteria (e.g., by polymyxins). 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 the resistance gene mcr-1 was also present in 15% of tested retail meat and in 1% of retrospective analyzed samples of human hospital patients from 2011 to 2014 (Ye et al., 2016). Since the first colistin resistance gene was discovered, additional genes mcr-2 to mcr-9 were identified in recent years (Carroll et al., 2019). These genes have been identified 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 (Sun et al., 2018).

Understanding the environmental fate of AMPs is extremely important, considering the biological activity of the AMPs and the risk of spreading antimicrobial resistance (Hurst et al., 2019; Menz et al., 2019). 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 (Menz et al., 2015). 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 (European Medicines Agency, 2017). 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 107, adsorption – desorption tests by OECD 106, ready biodegradability test by OECD 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 molecular structure (Fig. 1). 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.

2. Experimental section

2.1. Materials

Aqueous solutions were prepared with ultrapure water (> 18 M ± MΩ cm, Barnstead Nanopure Diamond system). The following reagents were all purchased from TCI Chemicals (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%), periphenethone (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 Döttingen, Switzerland. Furfuryl alcohol (Optima * LC-MS grade) was purchased from Fischer Chemicals. Suwannee River Fulvic Acid (SRFA, ZS101F) was purchased from the International Humic Substance Society. Standard soil types 2.1 (sand) and 6S (clay) were purchased from LUA Speyer and had the following soil characteristics: Soil 2.1: pH (0.01 M CaCl$_2$) 4.70 ± 0.01; 2.8 ± 0.7% clay content; and Soil 6S: pH (0.01 M CaCl$_2$) 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.

2.2. Sorption experiments

The experimental procedure was adapted from the OECD 106 guidelines: 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 Tables A1 and A2). 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 A3).

First, adsorption kinetics for one concentration of each of the test substances were assessed to determine the optimal contact time for the adsorption equilibrium (Fig. A1). Sacrificial samples (parallel method) were prepared, where 50 mg of soil and 1 mL of 10 mM CaCl$_2$ was added in duplicate vials for each kinetic sampling point and placed on horizontal shaker (250 rpm). After 12 h, 10 µM of the test compounds was spiked and vials were removed after 0, 4, 8, 24 and 30 h. Samples were centrifuged directly in the HPLC vial (5 min, 4000 g) using a micro-centrifuge (Centrifuge 5427 R, Eppendorf AG, Hamburg). Supernatant (300 µL) was transferred (conical glass insert inside a plastic centrifuge tube) and centrifuged again (10 min, 10,000 g). 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, including native soil pH, extractable extracellular enzymes and to account for potential loss of compound by sorption to the test vessels. Therefore, soil was contacted with CaCl$_2$ solution for 12 h before the supernatant was separated from the solids (centrifugation), spiked with test compounds, and analyzed for the stability of AMPs. Adsorption isotherm tests were performed using the same set-up with varied concentrations of the test compounds in triplicate ranging from 1 to 100 µM and an
incubation time of 6 h, which proved sufficient to achieve equilibrium.

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 290–400 nm as described previously (Davis et al., 2017). 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.

2.3.1. Reactivity with singlet oxygen

Daptomycin, polymyxin B, and polymyxin E were tested for their reactivity with singlet oxygen, ᵃ,O₂. Test compound (10 µM), the ᵃ,O₂ photosensitizer perinaphthenone (0.77 µM), and ᵃ,O₂ probe FFA (40 µM) were added to a solution of either H₂O or D₂O (approximately 90%). Samples were irradiated with UVA light centered around 360 nm on a merry-go-around Rayonet photoreactor, and aliquots were removed during exposure. The bimolecular reaction rate constant with ᵃ,O₂ was determined based on known kinetic solvent isotope effects for ᵃ,O₂ lifetimes as previously described (Davis et al., 2017). Bacitracin’s reactivity with ᵃ,O₂ was not included here because it has previously been studied (Lundeen et al., 2016). None of the AMPs underwent significant direct photodegradation under UVA irradiation during these experiments.

2.3.2. Transient absorption spectroscopy

We investigated further that the observed kinetic solvent isotope effect in the ᵃ,O₂ test with polymyxins did not result from a reaction with triplet excited state of perinaphthenone (³PN*) that we used as the ᵃ,O₂ photosensitizer. Therefore, transient absorption spectroscopy was used to monitor the lifetime of ³PN*. Experiments were performed in H₂O and D₂O 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 at the aqueous solubility limits and were sparged with a gas mixture of 5% O₂ with 95% N₂ starting 5 min before irradiation. The time-dependent change in absorbance (ΔA) for the triplet-excited state feature (³PN*, averaged from 479 to 495 nm) was monitored. Transient absorbance traces were fit to an exponential decay function for lifetime estimates, τ (1/kobs) (OriginPro 9.0, OriginLab Corp. Northampton, MA).

2.4. Biotransformation

The experimental procedure was adapted from the OECD 309 guideline: 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 17 °C), and July-August 2018 (average water temperature 18 °C) as previously described (Egli and Janssen, 2018). Channels were operated at a constant flow rate of 0.34 m/s 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, sus- pended 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 (120 °C) 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 (Table A4). 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 h 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, photo-transformation) AMPs were also spiked to nanopure water and sampled in a similar manner as described above. In addition, daptomycin and bacitracin were monitored for 30 h 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 × 100 mm; 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 A5). 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 z), maximum injection time: 50 ms, automatic gain control (AGC) target: 1.0 × 10⁵ and data dependent MS² (ddMS²) was collected with HCD collision energy: 35%, sweep gas of 1 (Arb). 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 h 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, photo-transformation) AMPs were also spiked to nanopure water and sampled in a similar manner as described above. In addition, daptomycin and bacitracin were monitored for 30 h 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.

2.6. 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 to 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 A1.). 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 considered (Table A6). For solutions in D₂O from the experiment to derive the bimolecular ¹O₂ reaction rate constants, H/D exchange of amino protons occurred and changes in the m/z were considered in the analysis (details in Text A2). To demonstrate that the ¹O₂ method based on 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-aminoquinolinyl-N-hydroxysuccinimidyl carbamate (AQC) prior to analysis by HPLC with fluorescence detection (details in Text A3, Fig. A3).

3. Results and discussion

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 (Kₐ values) were determined for two standard soils. Data in Fig. 2 show the AMP concentrations measured in the aqueous phase and the calculated concentrations in the soils. The Kₛ 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 (e.g., silicates, etc.) present net negative surface charges in soil and organic constituents (i.e., humic organic matter) can have a variable negative surface charge depending on the protonation state of its ionizable functional groups (i.e., carboxylic –COOH and phenolic –OH) (Bolan et al., 1999). Daptomycin is anionic at the soil pH values tested and the Kₛ values ranged from 20 to 40 L kg⁻¹ between the soils (Table 1). By normalizing the Kₛ values by the soil organic carbon content, the Kₒc 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 (Haigh, 1996). 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 (Thiele-Bruhn et al., 2004; Tolls, 2001).

The Kₛ values for the bacitracins were similar across the different variants but differed strongly between the soils. In the sandy, more acidic LUFa 2.1 soil, all bacitracin variants remained predominantly in the aqueous phase (i.e., lower Kₛ values) and the values increased by two orders of magnitude for the clayey, pH neutral LUFa 6S soil.
Overall, we observed a 30-fold difference in Kd values between these soils. While normalization to organic carbon content did not account for the difference in Kd values, normalizing to clay content reduced the difference between these soils to a 2-fold. At the corresponding pH values tested, bacitracin has a net cationic character with both carboxylic acid moieties being deprotonated (negatively charged), both primary amines being protonated (positively charged) and the histidine moiety being fully or partially protonated (positively charged, pKa ~ 6.9, Lundeen et al., 2016). Thus, soil pH and clay content 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 (Pinck et al., 1961a; Pinck et al., 1961b).

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 and 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 Kd values based on 1-point of the isotherm curve suggest strong sorption for polymyxin E1 (111 ± 36 L kg⁻¹) and E2 (256 ± 116 L kg⁻¹). An minimum limit of the Kd 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, TEF-FLON®), 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 polymyxin was stable (tested for LUFA 2.1 soil and polymyxin E). At environmental conditions, with pHs below 9, polymyxins are cationic.

Table 1

<table>
<thead>
<tr>
<th>AMPs</th>
<th>Adsorption to soil</th>
<th>Simulated sunlight</th>
<th>Singlet oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_d (L kg⁻¹)</td>
<td>k_d (direct)</td>
<td>k_d (indirect)</td>
</tr>
<tr>
<td></td>
<td>pH = 4.70</td>
<td>(Φ200-400 nm)</td>
<td>(×10⁻⁵ s⁻¹)</td>
</tr>
<tr>
<td></td>
<td>pH = 6.88</td>
<td>(×10⁻⁵ s⁻¹)</td>
<td>(×10⁶ M⁻¹ s⁻¹)</td>
</tr>
<tr>
<td>Bacitracin A</td>
<td>8 ± 2</td>
<td>n.d.</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Bacitracin B</td>
<td>6 ± 2</td>
<td>n.d.</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Bacitracin C</td>
<td>5.8 ± 0.5</td>
<td>n.d.</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>20.6 ± 0.8</td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.1³</td>
</tr>
<tr>
<td>Polymyxin B1</td>
<td>n.a.</td>
<td>n.d.</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Polymyxin B2</td>
<td>n.a.</td>
<td>n.d.</td>
<td>12.2 ± 0.5</td>
</tr>
<tr>
<td>Polymyxin E1</td>
<td>111 ± 36²</td>
<td>&gt; 52⁴</td>
<td>10.2 ± 2.5</td>
</tr>
<tr>
<td>Polymyxin E2</td>
<td>256 ± 116²</td>
<td>&gt; 185⁴</td>
<td>10.2 ± 2.5</td>
</tr>
</tbody>
</table>

a Literature values by Lundeen et al. (2016).
b Rate constant for indirect phototransformation only (direct k_d substracted from the total k_d).
c Determined using only 1 point from the isotherm.
d Minimum K_d 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.
surfactants, which tend to adsorb strongly to clays, colloidal materials, and organic matter (Haigh, 1996) and estimates of US EPA’s EPI Suite using the Molecular Connectivity Index Methods predict a log $K_{ow}$ value of 8.7 (EPA, 2012), 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 (Menz et al., 2019).

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 A7). 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 (Menz et al., 2015) 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.

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 mgC 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. (Fig. A4, UV–vis spectra of AMPs and light spectrum of solar simulator). Data in Fig. 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 a highly reactive PPRI, 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), which is comparable to environmental concentrations reported for surface waters (Page et al., 2010). Assuming diffusion controlled bimolecular reaction rate constants ($-1 \times 10^{6}$ 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 ($^{1}\text{O}_{2}$) is presented in more detail.

3.2.1. Reaction with singlet oxygen

The $^{1}\text{O}_{2}$ steady-state concentrations during the solar simulator experiment ranged from $1.8 \times 10^{-13}$ M (10 mgC. L$^{-1}$ SRFA) and these concentrations are representative for sunlit surface waters ($10^{-16}$−$10^{-12}$ M) (Shao et al., 1994; Wick et al., 2000; Wolfe et al., 1977, 1981). The bimolecular reaction rate constants with $^{1}\text{O}_{2}$ ($k_{\text{arxn}}$, 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 $^{1}\text{O}_{2}$ longer lived in D$_2$O and thus causes faster observed degradation in D$_2$O (Figs. A3 and A5) (Davis et al., 2018; Wilkinson et al., 1995). All AMPs tested showed significant reactivity with $^{1}\text{O}_{2}$ (Table 1). The reaction of $^{1}\text{O}_{2}$ 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 ($7.0 \times 10^{7}$ M$^{-1}$ s$^{-1}$ at neutral pH) (Chu et al., 2015; Lundeen et al., 2016). The bimolecular reaction rate constant for daptomycin with $^{1}\text{O}_{2}$ was $1.3 \times 10^{7}$ M$^{-1}$ s$^{-1}$ and thereby one order of magnitude slower than for polymyxins ($10.2$–$14.7 \times 10^{7}$ M$^{-1}$ 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.

![Fig. 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 mgC L$^{-1}$) for daptomycin (red triangles), bacitracins A1, B1, and C1 (superimposed blue circles), polymyxins B1 and B2 (super imposed green squares) and polymyxins E1 & E2 (superimposed purple diamonds). The singlet oxygen steady state concentration [$^{1}\text{O}_{2}$]$_{ss}$ ranged from 1.8 to $3.8 \times 10^{-13}$ 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). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](Image)
UVA light remained stable (Fig. A7B). Transient laser absorbance which was used to evaluate the $^{1}\mathrm{O}_2$ reactivity. No significant reactivity of polymyxin transformation products further supports that oxidation indeed took place. Data in Fig. 3B show the kinetic traces of the main transformation products observed for polymyxin E2 with $m/z$ mass shifts relative to the parent of $[+66]$, $[+96]$, $[+132]$, $[+162]$ and $[+192]$ (Tables A10–A11, extracted ion chromatograms, full-scan MS$^2$ spectra Figs. A9–A10). 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 alkylation.

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 $^{1}\mathrm{O}_2$ are tryptophan and kynurenine (Davies and Truscott, 2001; Nakagawa et al., 1977; Plowman et al., 2013). The bimolecular reaction rate constant of freely known transformation reactions of tryptophan and kynurenine with kinetic formation patterns. We observed several products as a result of known transformation reactions of tryptophan and kynurenine with $^{1}\mathrm{O}_2$, 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 ($[+80]$, Table A8) (Davies and Truscott, 2001; Nakagawa et al., 1977; Plowman et al., 2013). We tentatively identified transformation products of daptomycin from the reaction with $^{1}\mathrm{O}_2$ 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 $^{1}\mathrm{O}_2$, 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 ($[+80]$, Table A8) (Davies and Truscott, 2001; Nakagawa et al., 1977; Plowman et al., 2013). 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 $^{1}\mathrm{O}_2$, while more highly oxygenated transformation products ($[+60]$ and $[+80]$) were formed slower over the course of the experiment (Fig. A6).

A kinetic analysis of polymyxin transformation products further supports that oxidation indeed took place. Data in Fig. 3B show the kinetic traces of the main transformation products observed for polymyxin E2 with $m/z$ mass shifts relative to the parent of $[+66]$, $[+96]$, $[+132]$, $[+162]$ and $[+192]$ (Tables A10–A11, extracted ion chromatograms, full-scan MS$^2$ spectra Figs. A9–A10). 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 alkylation.

For polymyxins, the high reactivity with $^{1}\mathrm{O}_2$ 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 A3, Fig. A3). To exclude non-$^{1}\mathrm{O}_2$ reaction pathways that could contribute to the decay of polymyxins we conducted controls for abiotic hydrolysis, direct pho-tochemistry, and reaction with the ground state and triplet state sensitizer used to produce $^{1}\mathrm{O}_2$. Hydrolysis and direct phototransformation can be excluded as dark controls and controls without $^{1}\mathrm{O}_2$ sensitizer in UVA light remained stable (Fig. A7B). Transient laser absorbance spectroscopy was used to inspect potential reactivity with the triplet state of perinaphthenone and a potential kinetic solvent isotope effects, which was used to evaluate the $^{1}\mathrm{O}_2$ reactivity. No significant reactivity of polymyxin with triplet perinaphthenone was detected (Fig. A8 and Table A9).

Fig. 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; only for daptomycin), and a kinetic sorption test for up to 30 h 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Data in Fig. 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 (Fig. 4, green filled diamonds, solid lines). Comparable kinetics were observed for bacitracin B1 and C1 (Fig. A13). Daptomycin remained stable over 4 days exposure to the biofilm suspension that was inactivated by two cycles of autoclaving (Fig. 4, 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, after only one cycle of autoclaving of the biofilm suspension, we still observed decreasing concentration in the supernatant, which was also apparent for bacitracin (Fig. 4B, green open diamonds, dashed lines). These suspensions were, however, significantly less biologically active because benzoic acid, the positive control for biodegradation co-spiked with the AMPs, remained stable over 4 days. Our data also suggests that sorption to particulates in suspension was not dominating the removal process for daptomycin and bacitracins in the 1-time autoclaved biofilm suspensions. 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 autoclaved and underwent an additional freeze-thaw cycle as well as filtered biofilm suspension (particle-free). All bacitracin variants and daptomycin remained stable, indicating that neither sorption to particulate biofilm material nor transformation in the particle-free supernatant took place (Fig. 4, open green squares and orange triangles, respectively). Thus, we conclude that the degradation in the active biofilm suspension was in deep biologically mediated but 2-times autoclaving was necessary to obtain biologically inactive controls.

For polymyxins, the concentration of all variants decreased below the limit of quantification in the supernatant after 24 h of exposure to active biofilm suspensions, similar to benzoic acid. However, in the autoclaved controls, removal was only observed within the first 24 h and all concentrations remained constant and above the limit of quantification for the subsequent days (Fig. A14). The initial decrease of up to 86% after 9 h 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.

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 (Fig. 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 shift of [+16]) (D’Costa et al., 2012; Zumstein and Helbling, 2019). 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 (Kato et al., 2009). 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.

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} 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 modeling efforts. All AMPs also showed biotransformation potential by periphyton biofilm communities, which encourages future work to identify biotics transformation pathways.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

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References


