Citation for published version:

Prasse, C, Wenk, J, Jasper, JT, Ternes, TA & Sedlak, DL 2015, 'Co-occurrence of photochemical and microbiological transformation processes in open-water unit process wetlands', *Environmental Science & Technology*, vol. 49, no. 24, pp. 14136-14145. <https://doi.org/10.1021/acs.est.5b03783>

DOI:

[10.1021/acs.est.5b03783](https://doi.org/10.1021/acs.est.5b03783)

Publication date:

2015

Document Version

Early version, also known as pre-print

[Link to publication](https://doi.org/10.1021/acs.est.5b03783)

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Co-occurrence of Photochemical and Microbiological Transformation Processes in Open-Water Unit Process Wetlands

Carsten Prasse^{1,2}, Jannis Wenk^{1,3}, Justin T. Jasper¹,
Thomas A. Ternes², David L. Sedlak^{1,*}

¹ ReNUWIt Engineering Research Center and Department of Civil & Environmental Engineering,
University of California at Berkeley, Berkeley, California 94720, United States

² Department of Aquatic Chemistry, Federal Institute of Hydrology (BfG), Koblenz, Germany

³ Department of Chemical Engineering and Water Innovation & Research Centre (WIRC),
University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom

* Corresponding author: sedlak@berkeley.edu

To be submitted to Environmental Science & Technology

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Carsten Prasse^{1,2}, Jannis Wenk^{1,3}, Justin T. Jasper¹, Thomas A. Ternes², David L. Sedlak^{1*}

¹ ReNUWIt Engineering Research Center and Department of Civil & Environmental Engineering, University of California at Berkeley, Berkeley, California 94720, United States

² Department of Aquatic Chemistry, Federal Institute of Hydrology (BfG), Koblenz, Germany

³ Department of Chemical Engineering and Water Innovation & Research Centre (WIRC), University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom

*Corresponding author: sedlak@berkeley.edu

Abstract

The fate of anthropogenic trace organic contaminants in surface waters can be complex due to the presence of multiple parallel and consecutive transformation processes. In this study, the removal of five antiviral drugs (i.e., abacavir, acyclovir, emtricitabine, lamivudine and zidovudine) via both bio- and photo-transformation processes was investigated in laboratory microcosm experiments simulating an open-water unit process wetland receiving municipal wastewater effluent. The relative importance of the two transformation processes was strongly compound dependent. Phototransformation was the main removal mechanism for abacavir, zidovudine and emtricitabine with half-lives ($t_{1/2, \text{photo}}$) in wetland water of 1.6 h, 7.6 h and 25 h, respectively. In contrast, removal of acyclovir and lamivudine was mainly attributable to slower microbial processes ($t_{1/2, \text{bio}}$ = 74 h and 120 h, respectively). Identification of transformation products via high-resolution mass-spectrometry revealed that bio- and photo-transformation reactions took place at different moieties of the molecules. For abacavir and zidovudine, rapid transformation was attributable to the high reactivity of the cyclopropylamine and azido moiety, respectively. Despite substantial differences in kinetics of different antiviral drugs, biotransformation reactions mainly involved oxidation of hydroxyl groups to the corresponding carboxylic acids. Phototransformation rates of parent antiviral drugs and their biotransformation products were similar, indicating that prior exposure to microorganisms (e.g., in a wastewater treatment plant or a vegetated wetland) would not affect the rate of transformation of the part of the molecule that was susceptible to phototransformation. However, phototransformation strongly affected the rates of biotransformation of the hydroxyl groups, which in some cases resulted in greater persistence of abacavir and acyclovir phototransformation products.

Introduction

The discharge of municipal wastewater effluents into surface waters can result in the presence of trace organic contaminants at concentrations that pose potential risks to aquatic ecosystems and drinking water resources. After their release, many trace organic contaminants are attenuated by biological and photochemical processes. Although these processes often occur simultaneously or sequentially in the environment, most studies have considered the occurrence of only one transformation process at a time.¹⁻⁴ Thus, it is difficult to predict which transformation products are formed and whether or not transformation reactions occurring at one moiety alter the kinetics of subsequent transformation reactions. Furthermore, if partial transformation of a compound enhances the reactivity of other moieties, interaction of transformation processes could result in changes in the distribution of transformation products as well as their rates of removal. For example, carbamazepine, a compound that is particularly resistant to biotransformation is slowly transformed upon exposure to sunlight via direct photolysis and reaction with $\cdot\text{OH}$.^{5,6} This leads to the formation hydroxylated derivatives⁷ which are more easily biodegraded than the parent compound.⁸

Open water unit process wetlands have been developed as a polishing treatment step for municipal wastewater effluents.⁹ These managed natural systems utilize sunlight to remove trace organic compounds and deactivate pathogens.¹⁰⁻¹² In addition, microorganisms in the biomat formed at the bottom of these treatment basins reduce nitrate and contribute to aerobic biodegradation of trace organic contaminants.^{13,14} To assess the importance of the co-occurrence of biological and photochemical transformation reactions to reaction kinetics and product distribution, the fate of five antiviral drugs (i.e., abacavir, emtricitabine, lamivudine, zidovudine and acyclovir, see Figure 1) was studied under conditions comparable to those encountered in open-water unit process wetlands.

Antiviral drugs were chosen because they are widely used for the treatment of diseases such as herpes, hepatitis and HIV, and have been detected at concentrations above $1\text{ }\mu\text{g L}^{-1}$ in municipal wastewater effluents.¹⁵⁻¹⁸ No information about potential environmental effects resulting from the release of these compound into the aquatic environment is available so far. Furthermore, little is known about the effects of these compounds on

environmental viruses, a group of microorganisms that play a very important role in aquatic ecosystems.¹⁹

By investigating transformation kinetics and transformation mechanisms under conditions comparable to those encountered in open-water unit process wetlands it is possible to gain insight into how simultaneously occurring bio- and photo-transformation reactions affect the overall fate of antiviral drugs in sunlit surface waters. These compounds also serve as models for other families of compounds that contain moieties that are susceptible to bio- and phototransformation.

Figure 1. Antiviral drugs and their most likely sites of proposed photo- (□) and biotransformation (■) reactions.

Materials and Methods

Chemicals

Analytical reference standards of antiviral drugs and stable isotope-labeled analogues used as internal standards (purity > 99%) were purchased from Toronto Research Chemicals (Ontario, Canada). All other chemicals and solvents were obtained from Fisher Scientific (Fairlawn, NJ).

98

99 *Wetland water sampling conditions*

100 Phototransformation experiments were conducted in water collected from a pilot-scale
101 open-water unit process wetland located in Discovery Bay, CA. The facility treats about
102 10,000 gallons per day ($4.4 \times 10^{-4} \text{ m}^3 \text{ s}^{-1}$) of nitrified wastewater effluent from an adjacent
103 municipal wastewater treatment plant. Details about the open-water unit process wetland
104 were described previously.^{10,13} Water collected from the open-water wetland typically
105 contained 10 - 20 mg L⁻¹ -N NO₃⁻, 5 - 10 mg L⁻¹-C DOC, and 60 - 80 mg L⁻¹-C dissolved
106 inorganic carbon (HCO₃⁻ and CO₃²⁻). Samples for laboratory irradiation experiments were
107 collected from the mid-point of the wetland. All samples were filtered through pre-rinsed
108 1µm (nominal pore size) glass fiber filters (Whatman) and were stored in the dark at 4°C
109 until analysis, which occurred within 5 days.

110

111 *Laboratory photo- and biotransformation experiments (or: Determination of photo- and*
112 *biotransformation kinetics)*

113 Irradiation experiments were performed using a collimated beam Oriel Solar Simulator
114 (Spectra Physics, serial no. 91194) equipped with a 1000 W Xe lamp and either two
115 successive atmospheric attenuation filters (Spectra Physics, serial no. 81088 & 81017) or
116 one atmospheric and one UVB-filter (Spectra Physics, serial no. 81088 & 81050). Spectral
117 irradiance was routinely measured with a spectroradiometer (RPS 380, International light)
118 at different locations of the irradiated area to assess variability, which was always < 5%.
119 Details on lamp irradiance energies and the spectra of different configurations are given in
120 section 1.1 of the Supporting Information (SI). Irradiation experiments were carried out in
121 100mL black-painted glass beakers that were placed in a water bath at constant
122 temperature ($18 \pm 2^\circ\text{C}$). Initial concentrations of antivirals of approximately 0.5 µM were
123 used for all kinetics experiments. Pseudo-first order phototransformation rate constants of
124 antivirals and photochemical probe compounds used for the quantification of reactive
125 intermediates were calculated from the slopes of linear regression of the natural log of
126 concentration versus time. Control experiments in the dark revealed no degradation of
127 antiviral drugs indicating that their transformation in wetland water can solely be
128 attributed to photochemical processes.

For the elucidation of biotransformation kinetics experiments beakers were additionally supplemented with 10 mL of the biomat taken from the bottom of the pilot-scale wetland and kept in the dark (see Jasper et al, for further details).

Direct and indirect phototransformation. Experiments to assess direct phototransformation of antiviral drugs were conducted in buffered ultrapure water at pH-values ranging from 6 to 10 (pH 6 - 8: 5 mM phosphate buffer; pH 9 - 10: 5 mM borate buffer). Samples (1 mL) were collected at regular time intervals and stored at 4°C in the dark until analysis. Electronic absorption spectra of antiviral drugs at different pH values (see Fig. S2) were recorded with a UV-2600 UV-Vis Spectrophotometer (Shimadzu) using quartz-glass cuvettes (Hellma, Germany). Further details on determination of quantum yields using the *p*-nitroanisole (PNA)/pyridine(PYR) method²⁰ and related calculations are provided in section 1.6 of the SI.

Indirect phototransformation of antiviral drugs was investigated by the addition of specific quenchers to wetland water: *N,N*-dimethylaniline (DMA; 10 µM) was used to scavenge CO₃-radicals¹⁰, sorbic acid (2.5 mM) to scavenge excited triplet states of the dissolved organic matter (³DOM*)²¹, histidine (20 mM) to scavenge singlet oxygen (¹O₂)²² and isopropyl alcohol (IPA; 26 mM) to scavenge •OH-radicals.²³ In addition, experiments with specific photosensitizers and were conducted in ultrapure buffered water to determine reaction rate constants of antiviral drugs with individual reactive intermediates: For CO₃•⁻ either NaNO₃/NaHCO₃ or NaNO₃/duroquinone photosensitizer methods were used.^{24,25} The excited triplet state photosensitizers 3-methoxyacetophenone (3MAP) and anthraquinone-2-sulfonate (AQ2S) served as a proxy for ³DOM*.²⁶ Hydroxyl-radicals were generated by the irradiation of NaNO₃ solutions.²⁷ For ¹O₂ production Rose Bengal was used as a photosensitizer.²⁸ To further verify the role of ¹O₂, some experiments were performed in D₂O. Reaction rate constants were either determined by competition kinetics or by comparing reaction rates of antiviral drugs with those of established photochemical probe compounds (experimental details and calculations are provided in section 1.5 and 1.7). For all indirect phototransformation experiments, the concentration changes of photochemical probe compounds and antiviral drugs during irradiations were followed by HPLC-UV. Experimental and analytical details, including comprehensive results are provided in SI.

Given the structural similarities of antivirals with DNA bases, additional irradiation experiments were performed with adenine, 2-amino adenosine, cytosine, cytidine, guanine, thymidine and thymine (SI section 2.1.1) to obtain further information about the photoreactive moieties in the molecules and thus aid the identification of transformation products.

Identification of photo- and biotransformation products. High resolution mass spectrometry (HR-MS; LTQ Orbitrap Velos, Thermo Scientific, Bremen, Germany) was used to conduct accurate MS and MS/MS analysis of transformation products of antiviral drugs. To this end, experiments at elevated concentrations (40 μ M) were used. The LTQ Orbitrap Velos was coupled to a Thermo Scientific Accela liquid chromatography system (Accela pump and autosampler). HR-MS was conducted in the positive electrospray ionization (ESI) mode. To obtain structural information on the chemical structure of formed TPs, MSⁿ fragmentation experiments were conducted using data dependent acquisition. Further information on the applied setup and data dependent acquisition parameters can be found in the SI (section 1.2). Product formation of antiviral drugs in laboratory experiments was determined liquid chromatography tandem mass spectrometry (LC/MS/MS). Details on the analytical methods are provided in the SI (section 1.3).

Combined bio- and photodegradation experiments. The fate of antiviral drugs in the presence of sunlight and microorganisms was investigated over a 72 h period in the laboratory. Amber glass beakers (250mL) were filled with 180 mL of wetland water and 20 mL of freshly collected biomat material from the bottom of the Discovery Bay open-water unit process wetland. The experimental setup was the same as described above for photochemical experiments, but with three day/night cycles to simulate field conditions (8 h of daily irradiation followed by 16 h in the darkness; 72 h total). Antiviral drugs were added individually at concentrations of 0.5 μ M to ensure detection of both parent antiviral compounds and their transformation products. Samples were collected at regular time intervals and stored at 4°C in the dark prior to LC/MS/MS analysis, which occurred within 24 h. Further details about the analytical method can be found in the SI.

Results and Discussion

Phototransformation in wetland water

Phototransformation of the five investigated antiviral drugs in wetland water followed first-order kinetics ($r^2 \geq 0.98$; Figure S4-S8). In native wetland water (pH 8.9), the fastest phototransformation was observed for abacavir ($k_{\text{obs}} = 0.52 \pm 0.06 \text{ h}^{-1}$), zidovudine ($k_{\text{obs}} = 0.09 \pm 0.002 \text{ h}^{-1}$) and emtricitabine ($k_{\text{obs}} = 0.03 \pm 0.002 \text{ h}^{-1}$) whereas the transformation of acyclovir and lamivudine were significantly slower ($k_{\text{obs}} = 0.012 \pm 0.001 \text{ h}^{-1}$ and $0.011 \pm 0.001 \text{ h}^{-1}$, respectively) (Figure 2). No degradation of antiviral drugs in wetland water in the dark was observed indicating that their removal was solely attributable to photochemical processes. Photosynthetic activity leads to significant diurnal fluctuations of pH in open-surface wetlands.¹⁰ Therefore, phototransformation kinetics of antiviral drugs in wetland water were also determined at pH 6.5 and pH 10 (Figure 2).

Figure 2. Phototransformation kinetics of antiviral drugs in experiments with wetland water at different pH values and contribution of direct and indirect photolysis processes by comparison with results obtained in ultrapure water. Data for wetland water are corrected for light-absorption. Error bars show 95% confidence intervals.

Phototransformation of abacavir in wetland water increased when the pH value was adjusted to 6.5 or 10. This can be attributed to higher contribution of direct photolysis due to higher quantum yields at lower pH values (i.e. Φ_{app} is 4.2 – 11.4 times higher between pH 6 – 8, compared to pH 9 and 10, SI Table S5) and faster indirect photolysis at higher pH values. Comparison of transformation kinetics with results obtained in ultrapure water revealed the dominance of indirect photodegradation processes at pH 8.9 and 10, whereas direct photolysis was more important at pH 6.5. The addition of sorbic acid and histidine significantly reduced phototransformation rates of abacavir in wetland water (Fig. S4), suggesting the involvement of $^3DOM^*$ and 1O_2 in the photochemical fate of this compound. This was also supported by experiments with specific singlet oxygen and excited triplet state sensitizers (see below). Negligible removal of the structural analogues adenine and 2-amino-adenosine further revealed that the photolability of abacavir can be attributed to the cyclopropyl-moiety (see SI section 2.1.1).

Rates of phototransformation of zidovudine were not affected by changes in pH. Comparison with reaction rates in both ultrapure water and wetland water in the presence of scavengers revealed the dominance of direct photolysis (Fig. S5). Similar to abacavir, comparison with the depletion of structural analogues thymine and thymidine indicated that the azide moiety was responsible for the observed photoreactivity of zidovudine as both analogues showed no removal when exposed to light (see SI section 2.1.1).

Phototransformation of acyclovir in wetland water increased with increasing pH. Comparison with results from ultrapure water revealed that removal at pH 8.9 was solely due to indirect photolysis, whereas at pH 10 direct photolysis was also relevant. Significantly reduced rates of acyclovir phototransformation in the presence of sorbic acid and histidine indicated the importance of 1O_2 and $^3DOM^*$ to indirect photolysis (Fig. S6). In contrast to abacavir and zidovudine, phototransformation kinetics were similar to those observed for the structural analogue guanine (SI Fig. S15). Thus, phototransformation of acyclovir can be attributed primarily to the guanine moiety.

For lamivudine and emtricitabine, phototransformation kinetics in wetland water decreased with increasing pH. No removal of lamivudine was observed in ultrapure water indicating that its removal was entirely attributable to indirect photolysis. Higher

phototransformation rates of emtricitabine relative to lamivudine further indicated the strong influence of the fluorine atom for emtricitabine's photolability. The presence of the fluorine substituent leads to a higher absorption at 300-320 nm and thus alters the compound's UV absorbance at wavelengths between 300-320 nm (SI Fig. S2). Even though the absorption spectrum of emtricitabine did not change with pH, the quantum yield steadily decreases with increasing pH (Table S5). Phototransformation of lamivudine in wetland water was fully inhibited by sorbic acid, histidine and IPA but was unaltered in the presence of DMA (Fig. S7). This indicates the importance of $^3\text{DOM}^*$, $^1\text{O}_2$ and OH-radicals for its indirect phototransformation. For emtricitabine, phototransformation rates in wetland were only affected by IPA and sorbic acid (Fig. S8), indicating that reactions with $^1\text{O}_2$ are less important for this compound. The high photostability of its associated DNA base cytosine and nucleotide cytidine revealed the importance of structural modifications (thiol group (both compounds) and fluorine (emtricitabine)) to the observed photodegradability.

Additional experiments with individual reactive species revealed second order reaction rates with $\cdot\text{OH}$ at or above (abacavir, zidovudine) diffusion controlled levels ranging from $5 \cdot 10^9 - 1.1 \cdot 10^{11} \text{ M}^{-1}\text{s}^{-1}$ (Table 1). Antiviral compounds were reactive with $\text{CO}_3^{\cdot-}$, at rates between $1.2 \cdot 10^6 - 1.2 \cdot 10^9 \text{ M}^{-1}\text{s}^{-1}$, while only abacavir ($1.2 \cdot 10^9 \text{ M}^{-1}\text{s}^{-1}$) and acyclovir ($1.2 \cdot 10^7 \text{ M}^{-1}\text{s}^{-1}$) were obviously reactive with $^1\text{O}_2$. With the exception of abacavir, no depletion of antiviral compounds was observed in the presence of the model triplet photosensitizer 3MAP but in presence of AQ2S at rates similar or higher than the reference probe compound TMP, indicating a selective reactivity with excited triplet states. In order to check the plausibility of results for indirect phototransformation and obtain further indications for the role of different reactive species, steady-state concentrations of reactive species measured in wetland water (Table S4) were multiplied with measured second-order reaction rate constants of antivirals with $^1\text{O}_2$, $\cdot\text{OH}$ and $\cdot\text{CO}_3^-$, respectively (Table 1). Based on this estimation revealed for abacavir that at pH 8.9 approximately 60% of its indirect photodegradation in wetland water can be attributed to $^1\text{O}_2$. For acyclovir the important role of $^1\text{O}_2$ for photodegradation was also strengthened, although the prediction overestimates depletion rates by a factor two. For emtricitabine and lamivudine the

265 contribution of $^1\text{O}_2$, $^{\bullet}\text{OH}$ and $^{\bullet}\text{CO}_3^-$ can be assumed negligible, emphasizing the role of $^3\text{DOM}^*$
 266 and corroborating results of quenching experiments.
 267

Table 1. Quantum yields (pH 9) and apparent second-order reaction rate constants of indirect phototransformation of antiviral drugs via reaction with $^1\text{O}_2$, $^{\bullet}\text{OH}$, $^{\bullet}\text{CO}_3^-$ and excited triplet states (given relative to the degradation of the $^3\text{Sens}^*$ probe compound TMP). Quantum yields of antiviral drugs at pH 6-8 and pH 10 can be found in SI Table S5.

	[M Es ⁻¹]	[M ⁻¹ s ⁻¹]				[-]	
	Φ_{app} (pH 9)	$^1\text{O}_2$	$^{\bullet}\text{OH}$	$^{\bullet}\text{CO}_3^-$ ($\text{NO}_3^- + \text{HCO}_3^-/\text{CO}_3^{2-}$)	$^{\bullet}\text{CO}_3^-$ (DQ)	$^3\text{SENS}^*$ (AQ2S)	$^3\text{SENS}^*$ (MAP)
Abacavir	0.014 (±0.003)	1.2×10^9 (± 18%)	1.1×10^{11} (± 3%)	1.2×10^9 (± 4%)	- ^a	4.88	13.5
Zidovudine	0.45 (±0.15)	n.d.	1.3×10^{10} (± 2%)	2.4×10^6 (± 5%)	1.3×10^6 (± 4%)	0.62	n.d.
Acyclovir	0.01 (±0.005)	1.2×10^7 (± 25%)	5.0×10^9 (± 2%)	1.2×10^8 (± 2%)	6.3×10^7 (± 4%)	0.08	n.d.
Emtricitabine	0.016 (±0.005)	n.d.	9.3×10^9 (± 2%)	3.0×10^6 (± 4%)	4.3×10^6 (± 12%)	2.03	n.d.
Lamivudine	n.d.	n.d.	9.2×10^9 (± 1%)	1.2×10^6 (± 3%)	1.7×10^6 (± 3%)	1.86	n.d.

268 n.d.: not detected above level of uncertainty; ^a not applicable due to reaction of abacavir with DQ also in the dark

269

270 Comparison of photo- vs biotransformation rates

271 Dark experiments conducted with wetland water in the presence of biomat material
 272 indicated that biotransformation rates varied considerably among antiviral drugs.
 273 Biotransformation half-life times ($t_{1/2,\text{bio}}$) ranged from 74 h for acyclovir to 500 h (21 d) for
 274 emtricitabine (Fig. 3; Fig. S13). Under typical wetland treatment conditions (i.e., hydraulic
 275 retention times of 2-3 days), significant biological attenuation of acyclovir and abacavir is
 276 expected whereas for the other antiviral drugs removal via microbial processes is unlikely
 277 to be an important removal pathway. Comparison of transformation rates of antiviral drugs
 278 in the dark to those observed in irradiated wetland water indicated that
 279 phototransformation processes were dominant for abacavir, zidovudine and emtricitabine,
 280 while for acyclovir and lamivudine biotransformation was similar or more important than
 281 photolysis during typical summertime conditions (Fig. 3).
 282

Figure 3. Photo- and biotransformation rate constants k (h^{-1}) and associated half-life time $t_{1/2}$ (d) of antiviral drugs in laboratory experiments. Small bars within phototransformation columns indicate half-life times based on daily sunshine hours (9-15 hours). For the determination of biodegradation half-life times experiments were conducted in the presence of biomat in the dark. Error bars represent 95% confidence intervals obtained from linear regressions.

Transformation of abacavir

HRMS analysis indicated that four primary transformation products (TP318, TP288, TP284 and TP246) were formed during photolysis of abacavir in wetland water (SI section 2.2; Table S7). In agreement with results obtained for the structural analogues 2-amino-adenosine and adenine, fragmentation patterns of TP318, TP288 and TP246 revealed that the cyclopropylamine moiety was the main site of reaction, leaving the 2-amino-adenine (fragments: m/z 151.073, 134.046 and 109.051) and the 2-cyclopenten-1-methanyl moieties (fragments: m/z 95.353 and 79.054) unaltered.

Exact mass calculations of TP318 showed addition of two oxygen atoms to the cyclopropyl moiety (Δm +31.9898 Da). Results from MS² experiments were consistent with the scission of the cyclopropyl ring and the presence of a terminal hydroxyl group, as indicated by the cleavage of H₂O and CH₂O.

For TP288, MS data suggested modification of the cyclopropyl moiety via loss of one carbon atom and the addition of one oxygen atom, leading to the formation of an acetamide,

whereas TP246 was formed via cleavage of the cyclopropyl ring. The chemical structure of TP246 was confirmed by comparison with a commercially available reference standard. The exact mass and fragmentation pattern of TP284 was consistent with loss of two protons from either the cyclopropylamine or the 2-amino-adenosine moiety (fragments m/z 149.069 and 189.088 instead of m/z 151.073 and 191.104 compared to abacavir and the other TPs). Considering the high photolability of the cyclopropyl moiety, these structural changes were most likely due to the formation of a cyclopropylimine.

To assess the relative importance of direct and different indirect photolysis processes for formation of the observed abacavir transformation products, their formation was investigated in buffered water (direct photolysis only), wetland water (direct and indirect photolysis), and wetland water in the presence of different reactive intermediate scavengers. The results revealed that both direct and indirect photolysis of abacavir produced the same suite of TPs at similar relative concentrations, despite the fact that the disappearance of the parent compound was significantly accelerated in the presence of DOM and individual reactive intermediates (Fig. S17 & S18). Similar results were observed for irgarol, an algaecide that is structurally similar to abacavir, suggesting that the cyclopropylamine moiety is the main site of reaction under all conditions.²⁹ Photodegradation experiments in buffered ultrapure water with different optical filters indicated that wavelengths below 320 nm preferentially led to cleavage of the cyclopropyl moiety (TP246), whereas wavelengths above 320 nm (UV-A & visible light) led to scission of the cyclopropyl ring followed by partial oxidation (TP318) (Fig. S19).

These findings suggest that phototransformation of abacavir is initiated by a one electron oxidation of the cyclopropylamine moiety, leading to the formation of a cyclopropylaminium radical cation,^{30,31} followed by subsequent reactions resulting in the formation of various products. Interestingly, this phenomenon has also been utilized for the investigation of electron-hopping in DNA by modifying guanine and adenine with cyclopropyl moieties.^{32,33} Due to the instability of the initially formed closed ring radical cation, the modification results in rapid cyclopropyl ring-opening as well as 1,2-hydrogen migration, leading to the formation of an ionized allylamine.^{30,34} Scission of the ring is followed either by a complete cleavage of the cyclopropyl moiety (TP246) or reaction of the ring opened radical cation with H_2O/O_2 .^{32,34} In the latter case, electron release from the

carbon centered radical followed by hydrolysis leads to the formation of a 3-hydroxypropanaminium cation³⁵ and subsequent addition of water results in the formation of the 3-hydroxypropanamide (TP318). In our system, TP288 is formed by photolytic cleavage of the hydroxymethyl group which leads to the formation of the acetamide product.^{35,36} TP284 was most likely formed via H-atom abstraction, resulting in the formation of a neutral cyclopropyl radical followed by an electron transfer reaction and/or hydrolysis and elimination of water even though this reaction has only been shown to be catalyzed by enzymes so far.^{37,38}

Experiments with biomat material in the dark to determine the relative importance of biotransformation reactions indicated that microbial transformation of abacavir mainly occurred via oxidation of the primary alcohol group of the 2-cyclopenten-1-hydroxymethanyl side chain to produce the corresponding carboxylic acid (abacavir carboxylate, Fig. S13). This was consistent with previous experiments conducted with mixed liquor suspended solids from an activated sludge treatment plant.³⁹

When abacavir was exposed simultaneously to light and microorganisms (Fig. 4), a rapid loss of abacavir was observed during the first 8-hour light period (i.e., the initial concentration decreased by approximately 90 %). For the next 16 hours (i.e., the dark period) abacavir removal was significantly slower. When the light was turned back on, nearly all remaining abacavir disappeared. As expected, the light-induced transformation of abacavir gave rise to the four photo-TPs described above (middle panel of Fig. 4). The concentrations of these photo-TPs decreased by approximately 25% over the next 2.5 days, indicating that further transformation took place, either via photolytic or microbial processes.

Additional biodegradation experiments with the four photo-TPs of abacavir revealed that biotransformation occurs at the same moiety as observed for the parent compound, leading to the corresponding carboxylates (TP246 carboxylate, TP284 carboxylate, TP288 carboxylate and TP318 carboxylate; Fig. S20). Exact mass data and fragmentation patterns of bio-photo TPs determined by HRMS analysis are included in section 2.2 of the SI. Consequently, the observed decrease in concentration of photo-TPs shown in the middle

panel of Fig. 4 was mainly attributable to biotransformation, leading to a steady formation of carboxylate photo-TPs (bottom panel of Fig. 4). Faster transformation rates of abacavir photo-TPs observed during irradiation periods may have been attributable to enhanced biotransformation due to elevated oxygen concentrations or elevated pH values that occurred when the photosynthetic microorganisms in the biomat were active. Differences in biotransformation rates of TP246, TP284, TP288 and TP318, compared to abacavir (Fig. S14), indicate that alteration of chemical structure influences biotransformation kinetics, e.g. by affecting enzyme binding affinities or steric properties. Light-exposure of abacavir carboxylate formed in the dark led to its phototransformation, ultimately yielding the same photo-TPs as abacavir (bottom panel of Fig. 4). Considering that abacavir is already transformed extensively to abacavir carboxylate in activated sludge treatment,³⁹ a rapid elimination of both compounds can be expected in open-water unit process wetlands. In contrast to biotransformation reactions, similar phototransformation kinetics were observed for abacavir and abacavir carboxylate (Fig. S12). TP246 carboxylate was identified as the main product that accumulates over time because it is not susceptible to further reactions.

Figure 4. Transformation of abacavir (left, top) and resulting formation of photo-TPs (left, middle) and bio- / bio-photo-TPs (left, bottom)) as well as proposed transformation pathway (right) in combined in 3 day experiments in the presence of biomat with 8 hours of daily irradiation. In the transformation pathway, photo- and biotransformation reactions and structural changes in the molecules are indicated in orange and green, respectively.

Transformation of acyclovir

In contrast to abacavir, the transformation of acyclovir was dominated by microbial processes (Fig. 5), with biotransformation resulting in the formation of acyclovir carboxylate, which was not susceptible to further microbial transformation. These results are consistent with previous biotransformation experiments conducted with acyclovir in sewage sludge.⁴⁰

In the absence of biomat material, exposure of wetland water to simulated sunlight resulted in formation of two main photo-TPs (TP257 and TP223). HRMS analysis indicated that TP257 contains two additional oxygen atoms on the guanine moiety, as evidenced by

the detection of fragment m/z 184 instead of m/z 152 (Table S8; Fig. S16). Photosensitized degradation of guanine and guanosine occurs by reaction with excited triplet states, 1O_2 , $\cdot OH$ or $\cdot CO_3^-$.^{41,42} The main product of the reaction of guanine with 1O_2 has been identified as spiroiminodihydantoin.⁴³⁻⁴⁵ To assess the role of 1O_2 in the phototransformation of acyclovir in wetland water, experiments were conducted in both H_2O and D_2O in the presence of the 1O_2 sensitizer Rose Bengal (Fig. 5). Lifetimes of 1O_2 in D_2O are more than an order of magnitude higher than in H_2O ³⁸ and faster transformation of acyclovir in D_2O confirmed the role of 1O_2 for the indirect photolysis of acyclovir. In addition, the yield of TP257 increased in D_2O . Due to its photochemical properties acyclovir is likely to undergo self-sensitization via photoexcitation and subsequent formation of 1O_2 as shown for guanine and guanosine.⁴⁷⁻⁴⁹ For the second acyclovir photo-TP (TP223), HRMS analysis indicated the loss of two protons, most likely from the side chain, as evidenced by the detection of fragments m/z 152, 135 and 110, suggesting that the guanine moiety remained unchanged (Table S8). Additional information obtained from the fragmentation of the side chain was inconclusive but indicates oxidation of the terminal alcohol to the corresponding aldehyde via reaction with $\cdot OH$.⁵⁰

Results from 72h simulated sunlight experiments conducted in the presence of the biomat revealed a steady decrease of acyclovir during light and dark periods, indicating the dominance of biotransformation processes (Fig. 5b). However, biotransformation of acyclovir was significantly faster in the sunlight experiments compared to dark controls (Fig. 5a&b) suggesting that the higher oxygen concentrations and the elevated pH values that occurred when microorganisms in the biomat were undergoing photosynthesis played a role in the biotransformation processes.¹⁰ In the presence of simulated sunlight, production of the two phototransformation products (i.e., TP257 and TP224) was observed. No significant removal of TP257 was detected during dark periods, suggesting limited biotransformation via oxidation of the terminal hydroxyl-group of the side chain. Although the exact reason for this is unknown, a plausible explanation is that the structural modifications of the guanine core moiety prevented enzymatic oxidation of TP257. In contrast, concentrations of TP223 decreased in the dark. For the biotransformation product (i.e., acyclovir carboxylate), increasing concentrations were only observed during dark periods whereas its concentration decreased when exposed to sunlight. This indicates

that the compound was transformed further by photolytic processes, most likely via the same mechanisms as acyclovir. This was confirmed by additional irradiation experiments with acyclovir carboxylate in wetland water (results not shown).

Figure 5. Transformation of acyclovir in the presence of biomat in the dark (a) in combined photo- and biotransformation experiments (b), as well as formation of TP257 via reaction of acyclovir with 1O_2 in D_2O and H_2O using Rose Bengal as photosensitizer (c) and its proposed phototransformation pathway (d). The occurrence of acyclovir carboxylate at t_0 in (a) and (b) is due to its emission by the WWTP that feeds the wetland.

Transformation of zidovudine, lamivudine and emtricitabine

MS spectra of the phototransformation products of emtricitabine, lamivudine and zidovudine indicated structural changes at different positions on the molecules (Table S9-S11). For lamivudine and emtricitabine, HRMS analysis revealed oxidation of the riboside moiety (lamivudine TP245 and emtricitabine TP263), most likely via S-oxidation. This was confirmed by comparison with commercially available reference standards. Addition of H_2O to the 5-fluoro-cytosine moiety was observed for emtricitabine (emtricitabine TP265). Experiments conducted with the fluorine-free analogue lamivudine illustrates the importance of fluorine substitution: the F-moiety increases the light absorbance at

wavelengths > 300 nm (Fig. S2) for emtricitabine and leads to faster photodegradation (Figure 1, Table S5). Emtricitabine TP265 was formed via hydration of the double bond of the 5-fluorocytosine moiety, yielding a hydroxyl-group at position C6. For zidovudine, observed phototransformations were mainly attributable to the photolability of the azido moiety. Formation of zidovudine TP239 can be explained by cleavage of N₂, yielding a nitrene intermediate, which reacts further via intramolecular C-H insertion to an aziridine.^{51,52} Subsequent nucleophilic attack of the aziridine by water leads to the hydroxylation of the C atom in β -position or the formation of a hydroxylamine (zidovudine TP257).^{51,53} Results from HRMS analysis of zidovudine TP221 were inconclusive but indicated cleavage of N₂ and H₂O from the furanosyl moiety.

In addition, photolytic cleavage of the nitrogen-carbon bond between the DNA base moieties and the riboside analogue side chains was observed for all three compounds, resulting in formation 5-fluoro-cytosine (emtricitabine TP129), cytosine (lamivudine TP111) and thymine (zidovudine TP126). None of these TPs were detected in sunlight experiments in the presence of biomat (Fig. S21-22), indicating that they were rapidly transformed, most likely via microbial processes. For zidovudine, this was confirmed by additional biodegradation experiments with the photo-TPs (i.e., thymine, TP239, TP257), showing the rapid elimination of thymine (Fig. S22). Considering the importance of both thymine and cytosine as DNA building blocks, it is likely that they were incorporated into the microbial biomass. The fate of 5-fluorocytosine remains unclear. Similar to abacavir and acyclovir, biotransformation of emtricitabine, lamivudine and zidovudine was shown to result in the formation of carboxylated TPs via oxidation of the terminal alcohol as already observed for abacavir and acyclovir (Fig. S13). As carboxylated TPs are expected to follow the same phototransformation mechanisms as the parent compounds,, the interactions of photo- and biotransformation reactions is likely to result in the complete elimination of via mineralization and/or microbial uptake (Fig. 6).

Figure 6. Proposed photo- and biodegradation pathway of lamivudine (top), emtricitabine (middle) and zidovudine (bottom) in open-water wetland cells. Orange and green

arrows indicate photo- and biotransformation reactions,
respectively.

Environmental implications

The differences between kinetics and transformation product formation in presence and absence of the biomat highlight the complexity of transformation reactions that lead to the removal of trace organic contaminants in open water unit process wetlands and other sunlit waters. Attempts to predict the environmental fate of organic contaminants in these systems require an understanding of both processes as well as their potential interactions. Identification of TPs showed that bio- and phototransformation reactions take place at different positions of the antiviral molecules. Phototransformation of biodegradation products was found to occur at the same location as in the parent compound. As a result, mechanisms and kinetics were similar to those observed for parent antiviral compounds. This is important because carboxylate biodegradation products are typically present in much higher concentrations in biological treated wastewater compared to parent compounds.³² In contrast, biodegradation kinetics of phototransformation products of antiviral drugs differed substantially from that observed for the parent compound even though the site of enzymatic oxidation did not change. This can be explained by differences in enzyme affinities and steric hindrance. For example, phototransformation of acyclovir created a transformation product (TP257) that was not susceptible to biotransformation by microorganisms that could oxidize the parent compound in the dark. Combining kinetic studies with investigations of transformation product formation provides a better understanding of mechanisms relevant for the removal of trace organic contaminants in sunlit waters. By conducting biotransformation studies in the presence and absence of light it is possible to assess interactions between transformation processes and the likelihood that complete mineralization of trace organic contaminants will occur. These data also suggest that relative ratios of antiviral compounds and their transformation products might be useful as *in situ* probes to assess the relative importance of microbial and photochemical transformation pathways. This study also highlights the need to consider the formation of different transformation products in sunlit and light-shaded systems and the possibility of using knowledge of the reactivity of specific moieties

in chemical fate assessment. Considering the variety of formed transformation products, there is a need for appropriate risk assessment tools to assess potential adverse effects of transformation products with unknown toxicities on aquatic ecosystems. Finally, additional field studies are needed to further confirm the obtained laboratory results and to assess the suitability of the approach for the determination of the relative importance of individual transformation processes.

Supporting Information

Additional information on sample analysis; UV spectra of antiviral drugs; determination of indirect photolysis reaction rate constants, quantum yields, steady state concentrations of reactive intermediates in wetland water; experiments with DNA model compounds, MSⁿ fragments of transformation products; formation and fate of abacavir photo-TPs by different reactive intermediates; results of combined bio- and phototransformation experiments with emtricitabine, lamivudine and zidovudine.

Acknowledgement

C.P. was supported by a postdoctoral scholarship of the German Academic Exchange Service (DAAD), J.W. by a scholarship of the Swiss National Science Foundation (PBEZP2-142887). Financial support by the Engineering Research Center for Reinventing the Nation's Water Infrastructure (ReNUWIt) EEC-1028968, and TransRisk, funded by the German Ministry of Science and Education, is gratefully acknowledged.

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