Analysis, Occurrence and Fate of Antiviral Drugs in the Aquatic Environment

Analyse, Vorkommen und Verhalten von Antivirenmitteln in der aquatischen Umwelt

Dissertation

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SUMMARY

In the last two decades, pharmaceuticals have attracted broad scientific interest as they have been shown to be ubiquitously present in the aquatic environment. In particular, considerable concern has been raised regarding potential effects on human health due to the presence of pharmaceuticals in finished drinking water. Furthermore, research conducted so far indicates that transformations of pharmaceuticals in the environment as well as technical processes might result in the formation of potentially toxic transformation products.

Antiviral drugs are primarily used for the treatment of herpes, human immunodeficiency virus (HIV), hepatitis and influenza with prescribed amounts up to several tons per year. However, with the exception of oseltamivir and its active metabolite oseltamivir carboxylate, so far no information on their environmental behavior is available. Therefore, the aim of this thesis was to investigate the occurrence and fate of antiviral drugs in the urban water cycle, including the aquatic environment.

For the analysis of antiviral drugs in the aquatic environment a highly sensitive method was developed based on solid-phase extraction (SPE) followed by liquid chromatography tandem mass spectrometry (LC-tandem MS). Due to the wide range of physico-chemical properties, in particular with regard to the polarity of the analytes, two different approaches were investigated for their chromatographic separation, reversed-phase (RP) and hydrophilic interaction liquid chromatography (HILIC). Good chromatographic separation of individual antiviral drugs was achieved with both methods. However, due to the higher sensitivity, reproducibility and accuracy, the RP method was used for further analysis of antiviral drugs in environmental samples. The limits of quantification in raw and treated wastewater as well as surface water ranged between 1 (abacavir, oseltamivir, oseltamivir carboxylate) and 100 ng L^{-1} (ribavirin), 0.5 and 50 ng L^{-1} as well as 0.2 and 10 ng L^{-1}, respectively.

Subsequently, the developed method was applied to investigate the occurrence and elimination of antiviral drugs in wastewater treatment plants (WWTPs). With the exception of ribavirin all antiviral drugs were detected in WWTP influents with concentrations ranging between 5 and 2,000 ng L^{-1}. The comparison of concentrations in WWTP influents and effluents revealed a high elimination efficiency for acyclovir, penciclovir, stavudine, oseltamivir carboxylate, lamivudine and abacavir during wastewater treatment. For nevirapine, oseltamivir and zidovudine similar concentrations were found in WWTP influents and effluents. Nevertheless, most of the antiviral drugs were also detected in WWTP effluents indicating their emission into receiving waters. Consequently, the analysis of surface water samples revealed the widespread occurrence of antiviral drugs in concentrations up to 190 ng L^{-1} and 170 ng

L^{-1} for acyclovir and zidovudine, respectively. The widespread occurrence of antiviral drugs in surface waters was also demonstrated in a sampling campaign at the river Ruhr with 30 sampling points along the river. For most of the antiviral drugs analyzed higher concentrations were observed at sampling sites further downstream along the Ruhr. Furthermore, elevated concentrations downstream of WWTPs clearly showed the importance of WWTP effluents as point sources for the release of antiviral drugs into surface waters.

In addition, for the river Rhine, exceptionally high concentrations of oseltamivir compared to its active metabolite oseltamivir carboxylate were observed. This was unexpected as oseltamivir is extensively metabolized to oseltamivir carboxylate in the human body (> 75%), resulting in oseltamivir/oseltamivir carboxylate (OP/OC) ratios of 0.2-0.3. In surface waters OP/OC ratios between 0.3 and 3.0 were observed, attributable to the partial removal of oseltamivir carboxylate during wastewater treatment. In contrast to this, in the river Rhine significantly higher OP/OC ratios (OP/OC = 12-13) were observed, indicating the contribution of other sources in addition to urinary excretions of treated individuals. The analysis of samples taken at the international sampling station in Weil am Rhein, at the border between Germany and Switzerland, revealed a strong inhomogeneity in concentrations of oseltamivir and oseltamivir carboxylate across the river transect. Highest concentrations of oseltamivir (up to 200 ng L^{-1}) were observed in samples taken near the eastern riverbank, whereas it was completely absent in samples taken near the western riverbank. For oseltamivir carboxylate a similar trend was observed even though concentrations were significantly lower (up to 13 ng L^{-1}). In contrast to this, acyclovir, penciclovir and zidovudine were homogeneously distributed in all samples. Furthermore, the analysis of samples taken approx. 5 km upstream of the sampling station (near Basel) revealed the absence of both oseltamivir and oseltamivir carboxylate. This strongly suggested the inflow of waters containing high concentrations of oseltamivir and oseltamivir carboxylate in close proximity to the sampling station. Based on these findings, emissions from the pharmaceutical industry close to the sampling station Weil am Rhein could be assigned to be responsible for the high oseltamivir loads of up to 2 kg d^{-1}, which was finally confirmed by analysis of treated wastewater samples taken from the industrial WWTP in Basel.

A monitoring campaign during the swine flu pandemic in November/December 2009 in the Rhine near Koblenz revealed that the analysis of oseltamivir carboxylate in surface waters could be used to follow the course of flu epidemics. Based on the observed OP/OC ratios a differentiation between individual sources of oseltamivir and oseltamivir carboxylate in the river Rhine, namely urinary excretions of treated individuals and industrial discharges, was possible. During the monitoring campaign an intermittent contribution of both sources was observed with urinary excretions being primarily responsible for observed river loads during the time period with the highest

number of reported swine flu cases. Furthermore, acyclovir and zidovudine were detected in most of the samples in concentrations < 10 ng L^{-1}. Also for these compounds a high variation of river loads was observed with loads in general increasing with increasing river discharges, thus indicating the contribution of untreated wastewater discharges e.g. due to combined sewer overflows.

Due to the high concentration up to 2,000 ng L^{-1} in WWTP influents and the extensive elimination during wastewater treatment, the fate of acyclovir was investigated in greater detail. Laboratory batch experiments were conducted with sewage sludge taken from the aerobic nitrification compartment of the biological treatment unit from a conventional WWTP. To be able to characterize the influence of the chemical structure on the degradability of organic compounds and the formation of TPs, penciclovir, a guanine analogue closely related to acyclovir, was also investigated. To confirm the high elimination efficiencies of both compounds indicated from sampled WWTP influents and effluents, degradation kinetics were determined at environmental concentrations (4 µg L^{-1}). The experiments revealed first-order rate constants in relation to the amount of suspended solids (SS) of 4.9 and 7.6 L g_{SS}^{-1} d^{-1}, corresponding to half lives of 5.3 and 3.4 h for acyclovir and penciclovir, respectively. For a detailed investigation of the formation of TPs evolving from the biodegradation of both compounds, batch experiments at significantly higher analyte concentrations (150 mg L^{-1}) were conducted. The experiments revealed the formation of a single TP for acyclovir, whereas for penciclovir eight different TPs were observed. The elucidation of chemical structures of TPs via high resolution mass spectrometry (HR-MS) and nuclear magnetic resonance (NMR) spectroscopy exhibited that the transformation reactions taking place were restricted to the side chain of the molecules, leaving the guanine moiety unchanged. For acyclovir the oxidation of the terminal hydroxyl group of the side chain to the respective carboxylic acid, leading to the formation of carboxy-acyclovir (carboxy-ACV). The calculation of the mass balance of acyclovir revealed a closed mass balance for the transformation of acyclovir to carboxy-ACV in both batch experiments as well as in WWTPs, indicating the transferability of results from laboratory batch experiments to full-scale wastewater treatment. For penciclovir, in total eight different TPs were identified with the mass balance being closed until 120 h but decreasing afterwards to only approximately 30% until 240 h. Simultaneously, a decrease of the dissolved organic carbon (DOC) concentration was observed, thus indicating the partial mineralization and/or microbial uptake of penciclovir. However, in contrast to acyclovir, none of the penciclovir TPs could be detected in WWTP effluent samples, which can be primarily attributed to the low influent concentrations of penciclovir of only 50 ng L^{-1}.

Carboxy-ACV was detected in various surface waters in concentrations up to 3,200 ng L^{-1} indicating its widespread occurrence. Furthermore, it was shown that carboxy-ACV is also highly persistent in the environment as no removal was observed

in aerated batch experiments with soil. Consequently, carboxy-ACV was detected in oxic groundwater in concentrations up to 250 ng L^{-1}. However, the absence of carboxy-ACV in anaerobic groundwater indicates its degradability under anaerobic conditions. Nevertheless, carboxy-ACV was also detected in finished drinking water after bank filtration and sand filtration in concentrations up to 40 ng L^{-1}.

Due to its high persistence, the application of advanced treatment via ozone was investigated for its capability to remove carboxy-ACV from water. The experiments revealed a strong pH-dependence of ozonation reaction rates increasing by over three orders of magnitude from pH 1.7 to pH 8.5, thereby indicating the efficient removal of carboxy-ACV via ozone under ambient treatment conditions. However, the identification of oxidation products (OPs) revealed the formation of one main OP which could be identified via HR-MS and NMR as N-(4-carbamoyl-2-imino-5-oxoimidazolidin) formamido-N-methoxyacetic acid (COFA). Additional incubation experiments with sewage sludge revealed only an insufficient removal of COFA in biological treatment. Furthermore, COFA was shown to be toxic in a toxicity assay using *Vibrio fischeri* indicating that ozonation can result in the formation of toxic OPs. This might be of serious concern as COFA was detected in finished drinking water taken from a drinking water treatment plant applying ozonation and subsequent activated carbon treatment.

In summary, this study revealed the widespread occurrence of antiviral drugs in the aquatic environment. Furthermore, it could be shown that the elimination of pharmaceuticals in both biological and oxidative treatment do not necessarily result in their mineralization but rather leads to the formation of a variety of transformation and oxidation products. This is one of the first studies in which the fate and in particular the transformation of pharmaceuticals has been comprehensively investigated in almost the complete water cycle, from biological wastewater treatment to advanced oxidation processes via ozone. It was shown that the transformation of pharmaceuticals in the urban water cycle can ultimately result in the formation of toxic transformation products.

ZUSAMMENFASSUNG

Arzneimittel sind in den letzten Jahren in den Fokus des wissenschaftlichen Interesses gerückt. Dies liegt zum einen in ihrem verbreiteten Vorkommen in der Umwelt begründet, zum anderen aber auch in den sich dadurch möglicherweise ergebenden Auswirkungen auf den Menschen, da sie selbst in Trinkwasser nachgewiesen werden können. Darüber hinaus deuten aktuelle Studien darauf hin, dass Arzneimittel sowohl in der Umwelt als auch in technischen Aufbereitungsverfahren Vorläufer potentiell toxischer Transformationsprodukte sein können.

Antivirenmittel werden vor allem zur Behandlung von Herpes-, Hepatitis- und Grippeinfektionen sowie des Humanen Immundefizienz-Virus (HIV) eingesetzt. Bislang wurden diese jedoch, mit Ausnahme des Grippemittels Tamiflu®, in der Umwelt noch nicht untersucht. Somit war eine Aussage über eine mögliche Umweltexposition nicht oder nur bedingt möglich. Ziel dieser Studie war es deshalb das Vorkommen und Verhalten von Antivirenmitteln im urbanen Wasserkreislauf und der Umwelt näher zu untersuchen.

Um Antivirenmittel in der Umwelt nachweisen zu können wurde zunächst eine sensitive Analyenmethode entwickelt. Diese basiert auf der Extraktion der Antivirenmittel aus der Wasserphase mittels Festphasenextraktion (engl. *solid phase extraction*; SPE) und anschließender Detektion mittels Flüssigchromatographie-Tandem Massen-spektrometrie (engl. *liquid chromatography - tandem mass spectrometry*; LC-tandem MS). Aufgrund der großen Unterschiede in den physiko-chemischen Eigenschaften der Analyten insbesondere im Hinblick auf ihre Polarität wurden zunächst zwei unterschiedlichen chromatographische Trennungsverfahren untersucht: Umkehrphasen- (engl. *reversed phase*; RP) und hydrophile Interaktions-Flüssigchromatographie (engl. *hydrophilic interaction liquid chromatography*; HILIC). Mit beiden Verfahren konnte eine gute chromatographische Trennung aller Antivirenmittel erzielt werden. Die RP-Methode zeigte jedoch eine höhere Sensitivität, Reproduzierbarkeit und Genauigkeit, wobei die Bestimmungsgrenzen für alle Antivirenmittel in Rohabwasser, behandeltem Abwasser und Oberflächen unter 100, 50 bzw. 10 ng L^{-1} lagen. Aus diesem Grund wurde RP-Methode für die weitere Analyse der Antivirenmittel in wässrigen Umweltproben verwendet.

Die Untersuchung verschiedener Kläranlagen ergab, dass mit Ausnahme von Ribavirin alle Antivirenmittel in Kläranlagenzuläufen, also im unbehandelten Abwasser, in Konzentrationen bis 2 µg L^{-1} enthalten sind. Wie der Vergleich mit Kläranlagenausläufen zeigte, werden Antivirenmittel wie Acyclovir, Penciclovir, Abacavir oder Stavudine in der Kläranlage weitgehend eliminiert, wohingegen für Nevirapin, Oseltamivir und Zidovudin nur eine geringe Elimination beobachtet wurde.

Das Vorkommen der Antivirenmittel auch in Kläranlagenausläufen in Konzentrationen bis 560 ng L^{-1} zeigt jedoch, dass diese mit dem behandelten Abwasser in die Umwelt gelangen.

Folglich, konnten die Antivirenmittel auch in Oberflächengewässern in Konzentrationen bis 190 ng L^{-1} nachgewiesen werden. Die Bedeutung von Kläranlagen als wichtige Punktquellen für das Vorkommen von Antivirenmitteln in der Umwelt zeigte sich auch in einer entlang der Ruhr durchgeführten Messkampagne. Insgesamt wurden 30 Probenahmestellen, die von der Quelle der Ruhr bis zur Mündung in den Rhein reichten, beprobt. Für die meisten Antivirenmittel konnte eine Zunahme der Konzentrationen mit der Fließstrecke beobachtet werden. Erhöhte Konzentrationen nach Einleitungen von geklärtem Abwasser zeigten zudem die Bedeutung von Kläranlagen als wichtige Punktquellen für das Vorkommen von Antivirenmitteln in Oberflächengewässern. Die Analyse von Wasserproben aus dem Rhein ergab zudem für das Grippemittel Oseltamivir ungewöhnlich hohe Konzentrationen, insbesondere im Vergleich zu dessen aktiven Metabolit Oseltamivircarboxylat. Aufgrund der weit reichenden Metabolisierung von Oseltamivir zu Oseltamivircarboxylat (> 75%) im menschlichen Körper ergeben sich im Urin behandelter Personen Oseltamivir/Oseltamivircarboxylat (OP/OC) -Verhältnisse von 0,2-0,3. In Oberflächen-gewässern steigt dieses Verhältnis auf im Mittel 1,1 an, da Oseltamivircarboxylat im Gegensatz zu Oseltamivir teilweise in der Kläranlage transformiert werden kann. Für den Rhein hingegen ergaben sich signifikant höhere OP/OC-Verhältnisse (OP/OC ~ 13). Dies deutet, neben menschlichen Ausscheidungen, auf eine weitere Quelle hin. Eine zusätzlich durchgeführte Probenahme in der Internationalen Messstation in Weil am Rhein, an der Grenze zwischen Deutschland und Schweiz, ergab eine starke Inhomogenität der Oseltamivir- und Oseltamivircarboxylatkonzentrationen im Flussquerprofil. Wie sich zeigte, war Oseltamivir in den Proben die in der Nähe des östlichen Rheinufers entnommen wurden, in Konzentrationen bis zu 200 ng L^{-1} enthalten, während es in den Wasserproben vom westlichen Rheinufer nicht nachgewiesen werden konnte. Ein ähnliches Bild zeigte sich auch für Oseltamivircarboxylat, jedoch waren die Konzentrationen mit maximal 13 ng L^{-1} deutlich niedriger. Im Gegensatz dazu wurde für andere Antivirenmittel wie Acyclovir, Penciclovir oder Zidovudine eine homogene Verteilung im Querprofil des Rheins beobachtet. Zudem konnte in Wasserproben, die aus dem Rhein etwa 5 km flussaufwärts von der Messstation (bei Basel) entnommen wurden, weder Oseltamivir noch Oseltamivircarboxylat nachgewiesen werden. Somit konnten Einleitungen aus der pharmazeutischen Industrie als weitere Quelle für Oseltamivir und Oseltamivircarboxylat im Rhein identifiziert werden, was durch die Analyse von Proben aus der Industriekläranlage in Basel bestätigt werden konnte.

Eine während der weltweiten Schweinegrippepandemie im November und Dezember 2009 durchgeführte Monitoringkampagne im Rhein bei Koblenz belegte, dass die

Konzentration von Oseltamivircarboxylat, dem aktiven Metabolit von Oseltamivir, den Verlauf einer (Schweine-) Grippewelle widerspiegelt. Durch die Berechnung der OP/OC-Verhältnisse war es darüber hinaus möglich zwischen den beiden möglichen Quellen (menschliche Ausscheidungen und industrielle Einleitungen) von Oseltamivir bzw. Oseltamivircarboxylat zu unterscheiden. Zudem erlaubte die Frachtberechnung der beiden Stoffe auch eine Abschätzung der im Einzugsgebiet mit Tamiflu® behandelten Personen. Neben Oseltamivir und Oseltamivircarboxylat konnten auch das Herpesmedikament Acyclovir und das HIV-Medikament Zidovudin nachgewiesen werden. Die Konzentrationen lagen jedoch stets < 10 ng L^{-1}. Auch für diese beiden Antivirenmittel wurde eine hohe Variabilität der täglichen Frachten ermittelt, wobei in der Regel die höchsten Frachten an Tagen mit erhöhtem Abfluss beobachtet wurden. Dies deutet auf einen möglichen Einfluss der Einleitung von ungeklärtem Abwasser in Folge von Regenereignissen im Einzugsgebiet hin.

Aufgrund der hohen Konzentrationen von Acyclovir in Kläranlagenzuläufen von bis zu 2 µg L^{-1} und der beobachteten Elimination von >98%, wurde der Abbau von Acyclovir näher untersucht. Hierzu wurden Laborstudien mit Klärschlamm aus der biologischen Stufe (Nitrifikation) einer konventionellen Kläranlage durchgeführt. Um eine Aussage über den Einfluss der chemischen Struktur auf das Abbauverhalten und die Bildung von Transformationsprodukten (TPs) treffen zu können, wurde auch Penciclovir, ein dem Acyclovir strukturell sehr ähnliches Guanin-Analog, untersucht. Zunächst wurden die Abbaukinetiken beider Substanzen unter Verwendung umweltrelevanter Konzentrationen (4 µg L^{-1}) ermittelt um die in den Kläranlagen beobachteten hohen Eliminationsraten zu bestätigen. Die Experimente ergaben eine rasche Elimination von Acyclovir und Penciclovir mit Abbauraten (pseudo-erster Ordnung) von 4,9 bzw. 7,6 L g_{SS}^{-1} d^{-1}, was einer Halbwertszeit von 5,3 bzw. 3,4 h entspricht.

Um die Bildung und die Identität möglicher beim Abbau von Acyclovir bzw. Penciclovir gebildeter TPs detailliert untersuchen zu können, wurden zudem auch Experimente mit höheren Konzentrationen von bis zu 150 mg L^{-1} durchgeführt. Hierdurch konnte gezeigt werden, dass beim aeroben Abbau von Acyclovir eine einziges Transformationsproduktes gebildet wird, während für Penciclovir insgesamt acht TPs beobachtet wurden. Die Identifizierung der chemischen Strukturen der TPs mittels hochauflösender Massenspektrometrie und Kernresonanzspektroskopie (NMR) zeigte, dass sich die beobachteten Transformationen lediglich auf die Seitenkette der beiden Substanzen beschränkten, während die Guanin-Grundeinheit unverändert blieb. Für Acyclovir führte hierbei die Oxidation der terminalen Hydroxylgruppe der Seitenkette zur entsprechenden Carbonsäure zur Bildung von Carboxy-Acyclovir (Carboxy-ACV). Durch die Quantifizierung von Acyclovir und Carboxy-ACV war es zudem möglich, die Massenbilanzen sowohl in den Laborsystemen als auch in Kläranlagen zu berechnen. In beiden Fällen ergab sich eine geschlossene Massenbilanz was auch die Vergleichbarkeit der Laborergebnisse mit realen Bedingungen belegt.

Die Quantifizierung von Penciclovir und dessen TPs in den Laborsystemen ergab hingegen nur eine geschlossene Massenbilanz bis zu 120 h. im Anschluss ging diese bis auf ca. 30 % nach 240 h zurück. Da gleichzeitig auch eine deutliche Verringerung des Gehalts an gelöstem organischen Kohlenstoff beobachtet wurden, kann die nicht geschlossene Massenbilanz vermutlich auf die Mineralisierung und/oder die mikrobielle Aufnahme einiger Penciclovir TPs zurückgeführt werden. Aus diesem Grund und bedingt durch die Tatsache, dass Penciclovir in Kläranlagenzuläufen nur in Konzentrationen < 50 ng L^{-1} enthalten ist, konnte keines der Penciclovir TPs in Kläranlagenausläufen nachgewiesen werden. Carboxy-ACV wurde hingegen auch in einer Vielzahl von Oberflächengewässern in Konzentrationen von bis zu 3200 ng L^{-1} nachgewiesen. Auch in aeroben Batch-Experimenten mit Boden konnte kein signifikanter Abbau von Carboxy-ACV beobachtet werden. Dies deutet auf eine hohe Persistenz von Carboxy-ACV in der Umwelt hin . Als Folge konnte es auch in Wasserproben aus einem aeroben Grundwasserleiter in Konzentrationen bis 250 ng L^{-1} nachgewiesen werden. Die Abwesenheit von Carboxy-ACV in Proben aus einem anoxischen Grundwasserleiter deutet hingegen auf einen möglichen Abbau unter anaeroben Bedingungen hin. Dennoch konnte es auch in Konzentrationen bis 40 ng L^{-1} in Trinkwasser nachgewiesen werden, was auf eine unzureichende Entfernung in der Trinkwasseraufbereitung mittels Ufer- und Sandfiltration hindeutet.

Aufgrund der hohen biologischen Persistenz von Carboxy-ACV wurde dessen Entfernung mittels Ozonung näher untersucht. Wie sich zeigte, ist die Reaktion von Carboxy-ACV mit Ozon stark vom pH-Wert abhängig, wobei ein Anstieg der Reaktionsraten um drei Größenordnungen von pH 1.7 bis pH 8.5 beobachtet wurde. Dies lässt darauf schließen, dass Carboxy-ACV bei typischerweise in natürlichen Wässern vorkommenden pH-Werten aus Abwasser aber auch Trinkwasser sehr gut mittels Ozonung entfernt werden kann. Jedoch konnte gezeigt werden, dass es hierbei zur Bildung eines Oxidationsproduktes (OP) kommt. Mittels hochauflösender Massenspektrometrie und NMR konnte dieses OP als N-(4-Carbamoyl-2-Imino-5-Oxoimidazolidin)Formamido-N-methoxy-Essigsäure (COFA) identifiziert werden. Diese Substanz ist in der Literatur bislang noch nicht beschrieben worden, nicht einmal eine CAS-Nummer existiert. Batch-Experimente mit Klärschlamm zeigten, dass es in einer nachgeschalteten biologischen Behandlungsstufe nur zu einer unvollständigen Entfernung von COFA kommt. Zudem wurde für COFA in in Untersuchungen mit *Vibrio fischeri* eine Bakterientoxizität festgestellt was darauf hindeutet, dass es bei der Ozonung von Carboxy-ACV zur Bildung eines toxischen OPs kommt. Dies könnte auch direkte Auswirkungen auf den Menschen haben, da COFA in untersuchten Trinkwasserproben auch nach Ozonung und anschließender Aktivkohlebehandlung noch nachgewiesen werden konnte.

Zusammenfassend lässt sich sagen, dass die Verwendung von Antivirenmittel dazu geführt hat, dass diese heute in der aquatischen Umwelt weit verbreitet sind. Es

konnte zudem gezeigt werden, dass es beim Abbau von Antivirenmitteln in biologischen als auch in oxidativen Prozessen mittels Ozon zu keiner Mineralisierung sondern vielmehr zur Bildung von, teilweise stabilen, Transformations- bzw. Oxidationsprodukten kommt. Dies eine der ersten Studien in der das Verhalten, und insbesondere die Transformation, eines Arzneimittels (Acyclovir) nahezu im gesamten Wasserkreislauf, von der biologischen Abwasserbehandlung bis zu weitergehenden oxidativen Verfahren umfassend beschrieben werden konnte. Die Ergebnisse zeigen deutlich die Bedeutung biologischer Transformationsprodukte als möglichen Vorläufer toxischer Oxidationsprodukte.

CONTENT

1 General Introduction

1.1 Occurrence of pharmaceuticals in the urban water cycle and the environment

Anthropogenic organic compounds such as biocides and industrial chemicals are today ubiquitously present in the aquatic environment. The contamination of freshwater resources, especially groundwater, has become one of the major issues of our time. Considering the increasing water demand due to the predicted increase of the world population in the next decades, especially in parts of the world which are already today facing water scarcity such as Africa, this situation is even likely to deteriorate in the future. Also the environmental occurrence of pharmaceuticals has become an emerging issue.[1] The consumption of pharmaceuticals ranges from a few mg per capita per year up to more than one g per capita per year.[2, 3] As most (human) pharmaceuticals are not or incompletely metabolised in treated individuals, large fractions are excreted unchanged via urine or faeces.[4] In addition, also the disposal of unused medicines contributes to their release into the sewer system. All this leads to the presence of pharmaceuticals in the µg/L- or even mg/L-range in raw wastewater emphasizing the key role of wastewater treatment plants (WWTPs) for the release of pharmaceuticals into the environment.[5-7]

During conventional wastewater treatment (primary and secondary treatment), pharmaceuticals can undergo two main elimination pathways: i) sorption to sludge and the removal with the excess sludge and ii) (bio)degradation during biological treatment.[8-10] Volatilization is negligible due to the high polarity and low volatility of most pharmaceuticals. Consequently, the fate of pharmaceuticals strongly depends on their physico-chemical properties. As expected, the major sink for hydrophobic compounds such as miconazole and roxithromycin is the sorption to the sludge.[11-13] However, also these compounds can be emitted into the environment since sewage sludge is still used as a fertilizer in agriculture in many parts of the world.[14, 15] For polar compounds, in general, an elimination is only observed if they are degraded during biological treatment, i.e. either during nitrification or denitrification.[16-18] As a consequence, especially non-(bio)degradable polar compounds are likely to pass WWTPs and are thus emitted into the aquatic environment. In surface waters, in addition to (bio)degradation and sorption also photocatalyic degradation can play an important role, especially in shallow and clear surface waters.[21-23] Furthermore, particle-mediated transport, due to sorption to suspended particulates and the remobilized sediments, can contribute to the environmental distribution of pharmaceuticals.[19-20]

Even though the (bio)degradation of pharmaceuticals has long been believed to be associated with their complete mineralization, i.e. a conversion to CO_2 and H_2O, recent research has shown that this is rather the expection than the rule.[24-26] In fact, the apparent elimination is in most cases leading only to a transformation of the compounds and thus results in the formation of (often stable) transformation products. This is discussed in greater detail in chapter 1.3. As a result, transformation products of X-ray contrast media have been detected in groundwater and finished drinking water in concentration up to 4,600 and 500 ng L^{-1}, respectively.[25, 27]

Pharmaceuticals and their biological transformation products can be detected in surface water but also in groundwater and finished drinking water. This is of concern due to the unknown effects on the environment and human health.

1.2 RISKS ASSOCIATED WITH THE OCCURRENCE OF PHARMACEUTICALS IN THE ENVIRONMENT

Pharmaceuticals are typically observed in the environment in concentrations in the low ng/L-range. These low concentrations and the occurrence as mixtures rather than individual compounds makes an environmental risk evaluation of pharmaceuticals very difficult. The potential negative effects are diverse and can influence single individuals as well as whole populations. Furthermore, direct effects such as acute toxicity have to be distinguished from indirect effects such as impacts on the reproduction ability. Biological active compounds such as antibiotics have a direct impact on organisms if they are released into the environment.[28, 29] However, they can also indirectly influence humans if the chronic exposure of bacteria to these substances results in the formation of antimicrobial-resistant strains.[30-32] Synthetic estrogens used as contraceptives such as EE2, have been shown to be strong endocrine disruptors, which can lead to the feminisation of fish populations and thus the loss of the reproduction ability also at environmentally relevant concentrations in the low ng/L-range.[33-36] For diclofenac, a commonly used analgesic, which has been detected in surface waters in concentrations up to 1.2 µg L^{-1},[7, 21] histopathological alterations in kidneys and gills of rainbow trouts have been observed at environmentally relevant concentrations.[37, 38] An even more dramatic effect has been reported by Oaks et al. who showed that the decrease of vulture populations in Pakistan is directly linked to the concentrations of diclofenac in these animals.[39]

All effects discussed so far are associated with the occurrence of the pharmaceuticals themselves, i.e. the original active ingredients. However, as outlined above, most pharmaceuticals are metabolized in the human body or transformed after their release

into the sewer systems. With the chemical structures of the parent compounds being often only slightly modified, this leads to transformation products (TPs) with increased polarities which are likely to pose similar toxicological characteristics compared to the parent compounds.[40, 41] Therefore, also metabolites and transformation products have to be taken into consideration.[42] Furthermore, pharmaceuticals, as well as their metabolites and transformation products, can be precursors of toxic by-products which are formed during oxidative water treatment or disinfection of drinking water via ozone or chlorine.[43-45] The formation of N-nitrosodimethylamine (NDMA), a strong carcinogen, has been shown to be formed during chloramination, of compounds featuring dimethylamine moieties such as the histamine H2-receptor antagonist ranitidine.[46, 47] Chloramination is used as drinking water disinfectant especially in the USA. Schmidt and co-authors reported the formation of NDMA during ozonation of N,N-dimethylsulfamide (DMS), a microbial degradation product of the fungicide tolylfluanide.[48]

The understanding of the fate of pharmaceuticals, their metabolites and transformation products in the complete urban water cycle is crucial for a comprehensive risk evaluation.

1.3 STRATEGIES TO MINIMIZE THE RELEASE OF PHARMACEUTICALS AND THEIR TRANSFORMATION PRODUCTS INTO THE ENVIRONMENT

The occurrence of a large spectrum of pharmaceuticals in the environment clearly shows that conventional WWTPs are not capable of fully eliminating these compounds. Furthermore, the degradation of pharmaceuticals in wastewater treatment is in most cases not associated with a complete mineralization, thus leading to the formation of transformation products. Even though our knowledge is far from complete, the biological transformations observed so far have been dominated by a limited number of biochemical reactions such as hydroxylation, oxidation of hydroxyl groups to the respective aldehydes or carboxylic acids, N- and O-dealkylation or amide hydrolysis often leading to only small structural changes of parent molecules.[25-27, 49-56] This is not surprising as today's WWTPs have primarily been designed to minimize effluent nutrient loads, in particular nitrogen and phosphorous, to prevent eutrophication of receiving waters. Therefore, new strategies are needed to minimize the release of pharmaceuticals and their transformation products into the aquatic environment.

In the last years a lot of research has focused on new, advanced wastewater treatment technologies. Among these, ozonation and activated carbon treatment have already been shown to efficiently eliminate a large spectrum of pharmaceuticals also at full

scale.[57, 58] The great advantage of both techniques is that they can be directly implemented in existing wastewater treatment plants, for example as additional, so called advanced treatment.[58-60] Activated carbon has very high sorption capacities attributable to its enormous specific surface area which enables the removal of a large number of pharmaceuticals.[58, 61] However, as this technique is based on the sorptive removal of organic compounds, the activated carbon has to be reactivated or disposed (e.g. by incineration) after usage. In contrast, during ozonation organic compounds are degraded via chemical oxidation. Ozone (O_3) reacts specifically with electron-rich chemical moieties such as double bonds, activated aromatic systems and deprotonated amines.[62] Therefore, pharmaceuticals such as estrogens, beta blockers or macrolide and sulfonamide antibiotics are readily removed from treated waters by ozonation, whereas X-ray contrast media such as iopromide are only insufficiently eliminated.[63-66] In drinking water treatment, ozone is used for taste and odour control as well as for the removal of (pathogenic) microorganisms due to its strong disinfectant properties.[62]

However, O_3 is not stable in water and reacts with some water constituents, thereby decomposing primarily to OH-radicals which belong to the strongest oxidants in water. The stability of O_3 strongly depends on pH, alkalinity and amount and type of present organic matter.[67-71] OH-radicals, in contrast to O_3, react relatively unspecifically with organic compounds with degradation rate constants typically in the range of 10^7 to 10^9 M^{-1} s^{-1}.[71, 73] This is significantly faster than reaction rate constants typically observed for the reaction with O_3 (10^{-2} to 10^5 M^{-1} s^{-1}).[73-75] Consequently, during ozonation the reaction of both O_3 and OH-radicals have to be considered as compounds which are not or insufficiently attacked by O_3 might react with OH-radicals.

A main drawback of ozonation is that it does not lead to a complete mineralization of organic compounds but to the formation of oxidation products (OPs), which might be potentially toxic as they contain aldehyde, ketone or nitroso moieties.[62, 76-79] Therefore, one of the main challenges of the application of ozonation in water treatment is the limitation of the toxic by-product formation. A very promising approach to prevent the formation of toxic by-products is the combination of ozonation with biological treatment such as sand filtration or biological activated carbon treatment. A significant elimination of a large number of pharmaceuticals and other organic compounds was obtained together with a reduction of the overall toxicity of treated waters.[58, 59, 80-84] Furthermore, in advanced wastewater treatment, the ozonated water can be re-fed into the biological treatment step.[85, 86] The idea behind a combined oxidative-biological treatment is that compounds resistant to biological degradation can be oxidatively degraded via ozone resulting in the formation of oxidation products prone to microbial degradation. However, detailed information on the fate of specific compounds and their oxidation products is still lacking.

The biotic and oxidative treatment technologies discussed so far are not capable of efficiently eliminating all pharmaceuticals. Especially halogenated compounds such as iodinated X-ray contrast media and their biological transformation products have been shown to be recalcitrant in conventional but also in advanced treatment.[57, 61, 87] The presence of halogenated organic compounds is of considerable health concern due the toxicity of these compounds and their role as precursors of potentially toxic by-products formed during drinking water disinfection.[45, 88] Results from the degradation of chlorinated solvents, which are a major environmental issue as they have been emitted in large quantities e.g. from laundries and tanneries, revealed that these compounds can be degraded under anaerobic conditions via reductive dehalogenation.[89-92] However, the redox conditions typically observed in conventional WWTPs, e.g. in denitrification, are usually not favorable to enable reductive dehalogenation, making it necessary to significantly lower the redox potential as typically observed in denitrification (dissolved oxygen concentrations <1 mg/L; oxidation-reduction potentials ≥0 mV).[93-96] Results from membrane bioreactors (MBRs) indicate that lower redox potential (<0 mV) and thus reductive dehalogenation can also be obtained in full-scale plants.[97-99] Therefore, also anaerobic treatment can be used as a potential treatment method to efficiently degrade those compounds which are not or only insufficiently eliminated in aerobic and oxidative treatment. A prerequisite of the application of the anaerobic treatment techniques is the inhibition of the release of greenhouse gases such as methane into the atmosphere. This can be achieved either by the precise adjustment of the redox conditions to exclude methanogenesis and thus the formation of methane or by the collection of the gases which can, for example, be used for the production of electricity.[100-103]

Furthermore, a number of other promising techniques such as advanced oxidation processes (AOPs), membrane bioreactors, photocatalysis, non-thermal plasma and membrane filtration (e.g. nanofiltration, reverse osmosis) as well as electrochemical and photo-electrochemical methods have ben shown to eliminate a large spectrum of trace organic compounds, such as pharmaceuticals, from different water matrices even though this has so far been primarily investigated at laboratory or pilot scale.[104-116] The general drawbacks which all these approaches are facing are technical difficulties with regard to the scale-up for full scale water treatment and the limited cost competitiveness compared to existing treatment technologies, at least at the current state of knowledge and technology. However, this might change in the future.

Besides advances in the treatment of wastewater a number of other attempts try to tackle the problem even earlier by minimizing the release of pharmaceuticals into the communal sewer system. These mainly include the separation of urine with high loads of pharmaceuticals e.g. from humans treated with X-ray contrast media and the on-site treatment of wastewater with high loads of pharmaceuticals such as hospital or industrial wastewater.[117-120] However, these attempts are either associated with high

additional costs or with a lack in practicability, thus they will not be used in the near future.

Conventional wastewater treatment is not capable to efficiently remove polar organic micropollutants such as pharmaceuticals, making it necessary to develop new treatment strategies.

1.4 IDENTIFICATION OF UNKNOWN COMPOUNDS IN ENVIRONMENTAL MATRICES

The identification of unknown organic compounds is highly challenging considering the large number of anthropogenic chemicals emitted intentionally or unintentionally into the environment. This situation is even more complicated by the fact that i) most of these compounds are likely to undergo transformation reactions leading to the formation of an even larger number of TPs and ii) natural occurring compounds (natural organic matter; NOM) have to be distinguished from those which originate from anthropogenic activities.

For the sake of clarity, the different approaches used for the identification of unknown compounds are classified in the following in four principal categories (target analysis, suspect analysis, non-target analysis, effect-directed analysis; Table 1). However, this classification is not strict as there are often large overlaps. The individual approaches have advantages and disadvantages and none of them can be declared to be superior to the others. This is especially true as their applicability strongly depends on the underlying scientific question. For example, for a waterworks operator it might neither be realistic nor of interest to identify every single compound present in the feed water or the finished drinking water. Rather it might be more important to identify new emerging and toxic compounds which have previously not been detected, thus indicating, for example, a new contamination in the catchment area. Therefore, non-target analytical approaches can be applied to identify compounds which have not been present before by using a 'clean' feed water as reference. For regulators dealing with the approval of chemicals foreseen to be newly introduced into the market, it is essential to assess the potential risks associated with the release of these compounds into the environment. For an adequate risk assessment it is necessary to elucidate the fate and the degradation of compounds in the environment as well as to identify the formation of transformation products. This can be achieved by suspect analytical approaches. Finally, effect-directed analytical approaches can be used amongst others to assess whether the degradation of compounds showing toxic effects such as antimicrobials or biocides leads to a detoxification or whether formed transformation products are still of toxicological relevance.

TABLE 1. Analytical approaches for the elucidation of unknown organic compounds in environmental samples.

	Target analysis	Suspect analysis	Non-target analysis	Effect-directed analysis
Short description	Use of authentic standards to investigate their presence in the environment	Identification of unknown compounds (TPs) derived from known parent compounds	Screening of environmental samples for the identification of unknown compounds without any information on their chemical nature and (eco)toxicological effects	Identification of unknown compounds posing specific effects (e.g. mutagenicity, cytotoxicity, genotoxicity)
Advantages	Simple, relatively fast	Parent compound is known; use of stable isotope- and radio-labeled compounds; identification of TPs e.g. via NMR possible; quantification of TPs in the environment	No knowledge of chemical structures etc. needed	Direct assessment of toxicological relevant compounds (also applicable to mixtures not only individual compounds)
Disadvantages	Only applicable for known compounds for which a standard is available;	Time- and labor-intensive; parent compound has to be known	Identification of relevant compounds difficult; MS information often not sufficient for a final identification of organic compounds	Coupling of chemical and effect-directed analysis difficult; potential precursors of toxic by-products are not considered
Analytical approaches	GC- and LC-techniques	High-resolution mass spectrometry NMR	High-resolution mass-spectrometry	TLC-bioautography; toxicity assays; enzyme assays; bioaffinity profiling

Among the different approaches which can be used for the identification of unknown compounds such as transformation products of organic micropollutants, the use of authentic standards (**target analysis**) is the simplest and most straight forward. Its advantage is that the use of reference standards enables an exact quantification of the compounds in environmental samples.[5, 7, 121, 122] Thus, this is approach is especially useful for the analysis of compounds which can be assumed to be present in the environment, for example due to their high production volume or chemical stability. The main drawback is that the chemical compound to be investigated has to be available.

If a compound has been identified as environmentally relevant, laboratory batch experiments can be used to investigate its fate during wastewater treatment or in the environment.[24-27] This approach allows for the investigation of the degradability of the parent compound and the identification of formed transformation products (**suspect analysis**). To elucidate the biodegradation kinetics the target compound is usually spiked at environmentally relevant concentration (e.g. in the low µg/L-range). In order to elucidate the chemical structure of TPs, the same experiments are, in general, performed at significantly higher concentrations, typically in the mg/L- or even g/L-range (Fig. 1). One possibility thereby is the isolation of individual TPs by semi-preparative HPLC which is coupled to a fraction collector.[25, 26] Sterile control samples (e.g. obtained by autoclaving) can be used to assess the contribution of sorption but

also of abiotic reactions. However, possible alterations of the samples during sterilization have to be taken into consideration. To differentiate between organic compounds which are matrix constituents and those which are derived from the degradation of the compound of interest, blank samples are used. These samples are run in parallel but are not spiked with the target compound.

The elucidation of chemical structures can be obtained by a variety of mass spectrometry approaches such as MS^n experiments and different NMR techniques. Isolated TPs can be used as standards and thus also allow for a quantification in environmental samples for target analysis. This is also of importance for the calculation of mass balances not only in the batch systems but also in environmental samples to determine whether all relevant TPs have been identified and whether the same TPs are formed at environmentally relevant concentrations. In addition, the individual TPs can also be incubated in batch experiments to elucidate the (bio)transformation pathways.

The prerequisite and the main advantage of suspect analytical approaches is that the chemical structure of the parent compound is known, thus allowing, based on expert knowledge, for a first assessment of the TPs likely to be formed. This information can be used as a starting point for the elucidation of TPs via full-scan high-resolution mass spectrometry. The main disadvantage is that the suspect analytical approach in general only provides information of TPs formed from a single compound. Furthermore, for NMR relatively large amounts (in general several milligrams) of each TP in high purities have to be isolated, especially for ^{13}C- and ^{15}N-NMR. Therefore, it is in most cases necessary to incubate large amounts of the parent compounds. This can be particularly challenging for compounds which i) are likely to show adverse effects when applied in higher quantities such as biocides, ii) have a very low water solubility or iii) show different transformation processes at higher compared to lower concentrations.

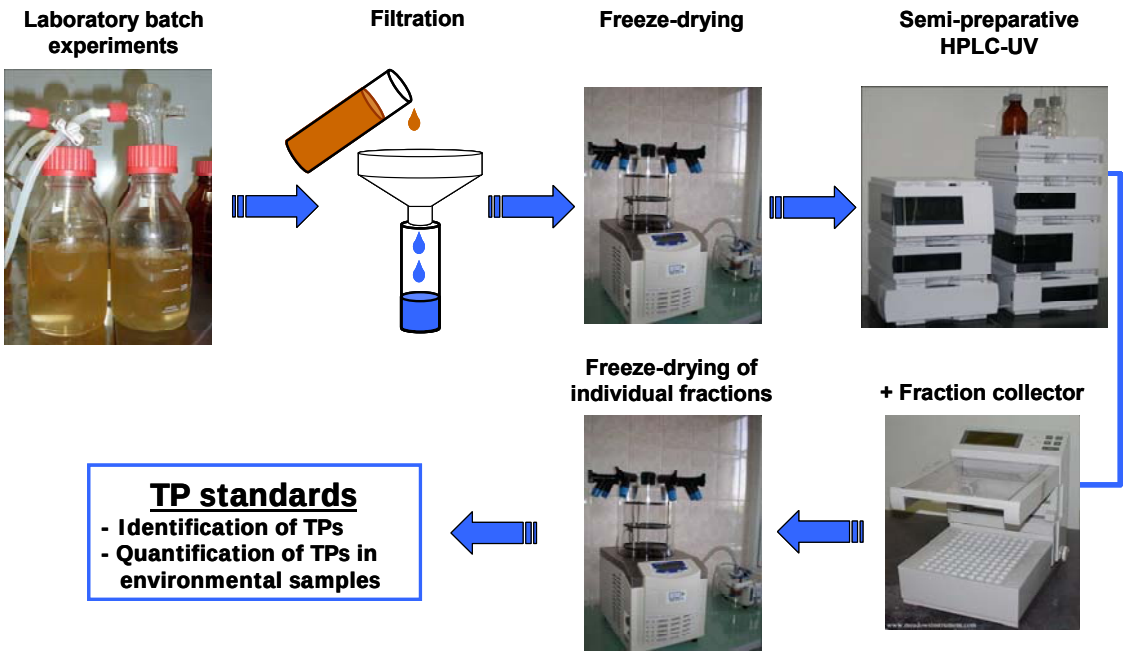

FIGURE 1. Experimental approach for the isolation of transformation products formed during the incubation of pharmaceuticals in laboratory batch experiments. The individual isolated TPs allows for their identification as well as for their quantification in environmental samples such as treated wastewater or surface water.

In addition to the approaches aiming at the elucidation of the identification of specific chemical substances and their TPs present in the environment, so called 'non-target' analytical techniques (**non-target analysis**) are becoming more and more important.[24, 123, 124] These techniques are strongly linked to recent advances in the development of analytical techniques, especially with regard to the possibility for a high-throughput screening which allow for the detection of several thousand unknowns in a single sample and in a single chromatographic run. They have originally been developed in the 'omics' -field in molecular biology such as genomics, metabolomics and proteomics.[36, 125-127] The term 'non-target screening' implies that the environmental samples such as surface water, groundwater, soil, sediment, etc. are screened for present compounds without any knowledge of their chemical structure. Thereby, it is important to note that only those substances which are accessible by the analytical technique used are considered. The application of high-resolution mass spectrometry enables the determination of exact masses and molecular formulae of organic compounds present in environmental samples. However, to obtain the true molecular formulae mass resolutions > 300,000 are necessary. Furthermore, it has been shown that even at a mass accuracy of < 1 ppm HR-MS might not be sufficient for exact determination of the molecular composition.[128]

To get hold of the large amount of data being generated, the acquired information is usually processed via different computational approaches which allows processing and analysis of large data sets as well as narrowing the number of compounds down to

those of relevance.[128-133] For the application of these techniques it is often necessary to make different assumptions and restrictions (e.g. molecular weight range, specifications of the molecular formula such as the presence of halogens or other heteroatoms). This is also one of the main drawbacks as the decision which compounds are of relevance and which are not is very challenging.

Other computational approaches which have been developed in the last years are used to facilitate the MS-based identification of unknown compounds. These mainly address the algorithm based processing of MS data, *in silico* fragmentation and MS database supported candidate search for metabolite identification.[134-137] Zhang and co-authors developed an algorithm for the background subtraction of high-resolution MS data (QTOF and LTQ Orbitrap).[138] The algorithm was applied to identify metabolites of pharmaceuticals in plasma samples and to record product ion spectra of the formed metabolites free of matrix interferences. Even though the comparison with reference spectra (e.g. NIST database) is a widely used tool in GC/MS for the identification of unknown compounds,[139, 140] liquid chromatography mass spectrometry (LC/MS) reference databases are only scarcely available. This is attributable to the fact that the fragmentation used in LC/MS strongly depends on the type of instrument (e.g. TOF-MS, QTOF-MS/MS, LC tandem MS, LTQ Orbitrap), fragmentation mechanism (energy induced vs. collision induced fragmentation) and fragmentation energies which are used.[141-143] This makes it generally necessary to conduct fragmentation experiments at various collision energies. Gómez-Ramos et al. created an accurate mass database of 147 compounds and their main fragments generated in CID MS/MS experiments.[144] Mixtures of up to 15 analytes were injected into a LC-QTOF-MS system in full scan as well as targeted MS/MS acquisition modes at four different collision energies to obtain accurate masses of parent ions and main fragments. The compiled database was then applied for the identification of transformation products of organic micropollutants, including pharmaceuticals, in WWTP effluents. In total, the authors were able to identify eight different TPs, including three which could additionally be confirmed by the use of analytical standards. A general drawback of the MS based approaches is that the obtained information is often not fully sufficient for an unambiguous identification but rather enables the proposal of potential chemical structure(s). As an example, the exact position of the hydroxylation of an aromatic ring cannot be elucidated via mass spectrometry techniques. Therefore, for a final confirmation of the chemical structures of unknown compounds the comparison with commercially available standards or the application of a second, independent identification technique (e.g. NMR) is essential.

Another set of computational tools which can be especially useful for non-target analytical but also for suspect analytical approaches are biodegradation prediction systems such as the University of Minnesota Pathway Prediction System (UM-PPS) or Catabol. These allow for the prediction of biotransformation pathways of organic substances.[145-147] The basis of these systems are large databases in which biochemical

reactions which have been reported in literature regarding the degradation of specific organic compounds, are compiled. For the prediction of TPs, the functional groups in the target compound are compared to those stored in the database to identify those which are potential targets of microbial catabolic reactions for which then the transformations are predicted. This information can then be used to screen environmental samples for predicted TPs. [24, 81, 148-150]

The general restriction of the methods discussed so far, aiming at the identification of unknown compounds, is that they do not deliver any information on the potential (eco)toxicological effects. The presence of specific functional groups, such as aldehydes can only give an indication of the potential (eco)toxicity. Approaches to overcome this restriction are so called effect directed approaches (**effect directed analysis**; EDA).[151, 152] As indicated from its name, EDA aims at the identification of compounds which show (eco)toxicological effects.[153-155] Therefore, the advantage of EDA is that the large number of compounds present in environmental samples is reduced to those which might be, due to their toxicity, of environmental concern. In addition, these approaches can also be directly coupled to analytical approaches in order to identify the chemical structure of the toxic compounds. Schulze et al. investigated the formation of phytotoxic transformation products formed during the photodegradation of diclofenac by using the green algae Scenedesmus vacuolatus.[156] The authors used peak directed reversed-phase HPLC fractionation of the solid phase extract to obtain six fractions, which were then individually tested for their toxicity. One of the fractions showed an increased toxicity compared to the parent compound. This could be attributed to the formation of the phototransformation product 2-[(2-chlorophenyl)amino] benzaldehyde (CPAB), which was identified via GC/EI/MS analysis in full-scan mode. High performance thin-layer chromatography (HPTLC) with bioactivity screening (using *Vibrio fischeri*) and subsequent MS analysis can been used to identify toxic compounds which are present in environmental samples and to elucidate their chemical structure.[157, 158] The samples are sprayed on a HPTLC plate which is then developed by using a single solvent or automated multiple development (AMD). AMD allows for the use of several different solvents to obtain the chromatographic separation of individual compounds. The developed plate is then immersed into a *Vibrio fischeri* solution and the bioluminescence of the plate is monitored using a CCD camera. Black spots on the plate thereby indicate the presence of toxic compound(s). The individual compounds can then be extracted from the HPTLC plate using an MS interface to elucidate the chemical structures of the toxic compounds.

Bioaffinity profiling and enzyme activity profiling are approaches which have so far been primarily used in molecular biology and medicine.[159, 160] Bioaffinity profiling is a microplate-based high-throughput screening methodology using on a chromatographic separation with subsequent MS detection and an on-line bioassay. Hogenboom and

co-authors used a continuous-flow analytical screening system by applying electrospray mass spectrometry to measure the interaction of biologically active compounds with soluble affinity proteins.[161] After chromatographic separation the compounds were allowed to react with the affinity protein (streptavidin) in a continuous-flow reaction system. Afterwards, a reporter ligand (fluorescein-labeled biotin) was added to saturate the remaining free binding sites of the affinity protein. The concentration of the unbound reported ligand was determined via electrospray MS in MRM mode. The presence of an active compound is indicated by an increased concentration of the unbound reporter ligand.

Similar to bioaffinity profiling, enzyme activity profiling allows for the direct screening of enzyme inhibiting compounds in environmental samples. This approach was successfully applied by Akkad et al. for the detection of insecticides in water samples using rabbit liver esterase (RLE), *Bacillus subtilis* esterase (BS2), and cutinase from *Fusarium solani pisi* (CUT).[162] Staining was performed with Fast Blue Salt B (3,3'-dimethoxy-4,4'-biphenylbis(diazonium) chloride). The reaction of Fast Blue Salt B with α-naphthol, which is enzymatically released from α-naphthol acetate used as substrate, resulted in a violet background while zones of inhibitors remain colorless due to lack of substrate conversion.[163] After sample application and chromatographic separation using AMD, the HPTLC plates were immersed into an enzyme solution and the absorbance was measured at 533 nm. For identification of insecticides, the inhibition zones were extracted with a TLC-MS interface. The drawback of EDA is that it generally allows only for the simultaneous testing of a single toxicological endpoint such as cytotoxicity. Therefore, for an appropriate risk assessment of individual compounds, several different tests are necessary to address, for example, mutagenicity, genotoxicity, or endocrine disruption. Compounds which are precursors of toxicological relevant substances such as NDMA are not considered in EDA.

An important point which should always be taken into consideration, especially when using laboratory batch studies, is that also the design of the experiments can give useful information not only to the identification of TPs but also to the conditions under which they are formed. The addition of specific electron acceptors such as nitrate, sulfate, manganese (Mn(IV)) or iron (Fe(III)) can be used to investigate the influence of redox conditions on transformation reactions taking place.[164-166] Furthermore, the addition of substrates or inhibitors allows for the identification of relevant microorganisms responsible for the observed transformations. As an example, allylthiourea, a known inhibitor of nitrification, can be used to determine the impact of nitrifying bacteria.[167, 168] Finally, stable isotope labeled (e.g. ^{13}C, ^{15}N) and radioactive labeled compounds (^{14}C or ^{3}H) can provide valuable information on the fate of specific organic compounds. Stable isotope labeled compounds are especially useful for the MS-based identification of TPs as the isotopic signature allows the following of the formation of TPs enriched with ^{13}C.[169, 170] The great advantage of radioactive labeled

substances is that a complete mass balance can be determined, including those proportions which are mineralized or irreversibly bound or incorporated to solid particles or biomass.[171-174]

A variety of state-of-the art techniques and experimental setups allow for the identification of unknown compounds via target, suspect, non-target and effect directed analytical approaches.

1.5 ANTIVIRAL DRUGS

Antiviral drugs are a group of pharmaceutical active compounds used for the treatment of a variety of viral infections, including herpes and hepatitis viruses, human immunodeficiency virus (HIV) and influenza viruses. Acyclovir was the first drug approved for treatment of herpes infections in the 1970s.[175-177] All antiviral drugs in use today only prevent the replication of viruses but they do not kill them. Thus they are termed virostatics. Viricides, capable of destroying viruses, are currently not available on the market. The underlying principle of the mode of action of antiviral drugs is that they interact at different stages of the life cycle of viruses, mainly by interacting with specific proteins (Table 2). Therefore, they have to be highly specific as proteins also play an essential role in the hosts of viruses. Otherwise their usage would inevitably lead also to a weakening of the immune system of the host and thus to the expansion of the viral infection. As an example, the mechanism of the interaction of acyclovir with viral DNA is shown in Fig. 2.

TABLE 2. Mode of action and target viruses of antiviral drugs investigated in this thesis.

Mode of action	Target virus(es)	Approved antiviral drug(s)
Viral DNA polymerase inhibitors	Herpes viruses	**Acyclovir, penciclovir**
Reverse transcriptase inhibitors	Human immunodeficiency virus	Non-nucleoside reverse transcriptase inhibitors: **zidovudine, stavudine, lamivudine, abacavir** Nucleoside reverse transcriptase inhibitors: **nevirapine**
Viral neuraminidase inhibitors	Influenza A and B virus	**Oseltamivir**
Inosine 5'-monophosphate dehydrogenase inhibitors	Hepatitis C virus and respiratory syncytial virus	**Ribavirin**

Due to their high specificity, the potential effects resulting from the release of antiviral drugs into the environment are difficult to assess. This is especially true as our knowledge of the occurrence of specific viruses in the environment is still very scarce, which can primarily be attributed to difficulties associated with their analysis. So far it has been shown that viruses are the most numerically abundant biological entities on earth and it has been estimated that oceans contain more than one million viruses per milliliter.[178] Furthermore, viruses play an essential role in the aquatic ecosystem with virioplankton being the most diverse component of plankton.[179-181] Therefore, the release of antiviral drugs into the environment may have effects on the whole aquatic ecosystem.

For anti-influenza drugs such as oseltamivir and its active metabolite oseltamivir carboxylate, considerable concern has been raised regarding the potential development of viral resistances in wildfowl.[182, 183] Birds are competent hosts of influenza viruses, therefore, the chronic exposure to antiviral drugs might have serious implications also with regard to human health.[184, 185] This is especially true during flu pandemics with predicted environmental concentrations (PEC) of oseltamivir carboxylate up to 80 µg L^{-1} in surface waters.[186] Even though so far no toxic effects were observed for oseltamivir and oseltamivir carboxylate,[187-189] it has been shown that high loads of these antiviral drugs can have an influence on the bacterial community in both WWTPs and the environment.[190, 191]

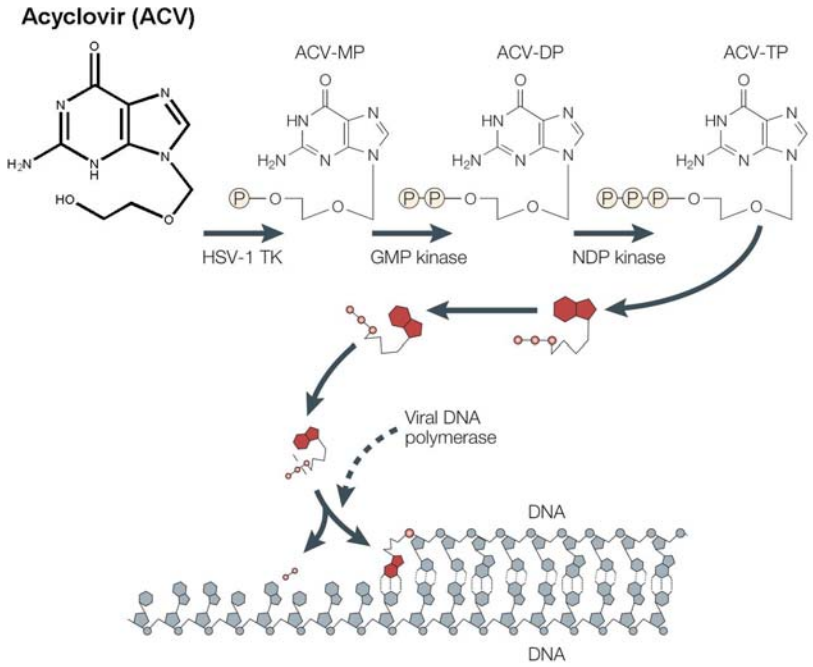

FIGURE 2. Mechanism of the interaction of acyclovir (ACV) with viral DNA (modified from ref. 177). Abbreviations: ACV-MP/DP/TP, ACV-monophosphate/ diphosphate/triphosphate; HSV-1 TK, Herpes virus-encoded thymidine kinase; GMP, guanosine monosphosphate; NDP, nucleoside 5'-diphosphate.

During conventional wastewater treatment oseltamivir carboxylate is only insufficiently eliminated, whereas a substantial removal can be achieved by subsequent ozonation.[192-194] Furthermore, it has been shown that ozonation also results in the loss of the neuraminidase activity of oseltamivir carboxylate.[195] After the release into the environment oseltamivir and oseltamivir carboxylate have been shown to be only poorly degraded.[196, 197] As a consequence, oseltamivir and oseltamivir carboxylate have been detected in surface waters in concentrations up to 200 ng L^{-1}.[194, 198]

Antiviral drugs are a class of pharmaceuticals which are likely to be present in the environment with so far widely unknown consequences for the environment but also for human health.

1.6 OBJECTIVES

The overall objective was the investigation of the occurrence and fate of antiviral drugs in the urban water cycle and the environment.

For this it was necessary to develop a highly sensitive analytical method (**Chapter 2**). The challenge was to enable the accurate analysis of antiviral drugs in a variety of aqueous matrices such as WWTP influents and effluents, surface waters, groundwater and drinking water at concentrations in the low ng/L-range. The developed method was then applied to investigate i) the elimination efficiencies of antiviral drugs during conventional wastewater treatment and ii) the environmental occurrence of antiviral drugs at a point of no pandemic outbreak.

Another aim was to examine the occurrence of anti-influenza drugs such as Tamiflu® in surface waters during (swine) flu pandemics (**Chapter 3**). Furthermore, it should be assessed whether surface water concentrations of anti-influenza drugs can be used to follow the course of a swine flu epidemic in surface waters. The simultaneous analysis of other antiviral drugs thereby allows evaluation of their occurrence in surface waters over a longer time period.

To investigate the influence of biological transformation processes on the fate of antiviral drugs in wastewater treatment and the environment the degradation of acyclovir and penciclovir were investigated in greater detail (**Chapter 4**). The main aims were to i) identify evolving TPs and ii) elucidate the biotransformation pathways. The analysis of environmental samples was used to assess the occurrence and fate of TPs in the environment.

The final task was to investigate the applicability of ozonation as an additional treatment step for its capability to remove antiviral drugs from wastewater as well as drinking water (**Chapter 5**). Therefore, acyclovir and its stable biological transformation product carboxy-acyclovir were investigated in greater detail to i) investigate the pH-dependency of reaction rate constants for the reaction with ozone, ii) identify evolving OPs and iii) determine the (bacterial) toxicity of OPs. Furthermore, it was investigated whether OPs can be further degraded in subsequent biological treatment and whether similar OPs are formed from guanine and its derivatives such as guanosine.

1.7 THESIS OUTLINE

Development and application of a highly sensitive method for the analysis of antiviral drugs in the aquatic environment.

Chapter 2 describes the development, validation and application of an analytical method based on solid-phase extraction (SPE) and LC-tandem MS detection for the analysis of antiviral drugs in the aquatic environment. The developed method was applied to investigate the occurrence of antiviral drugs in wastewater treatment plants as well as surface waters.

Occurrence of antiviral drugs and in particular anti-influenza drugs in surface waters during a (swine) flu pandemic.

Chapter 3 reports the occurrence of anti-influenza drugs such as oseltamivir in surface waters. The ratio of oseltamivir to oseltamivir carboxylate was thereby used to assess the influence of the potential sources, namely excretions from treated individuals and emissions from the pharmaceutical industry. Furthermore, the analysis of other antiviral drugs was used to investigate their occurrence in surface waters over a longer time period.

Transformation processes in biological wastewater treatment.

Chapter 4 describes the degradation of acyclovir and penciclovir in biological wastewater treatment. Biodegradation kinetics were determined and formed TPs were identified using high-resolution mass spectrometry and NMR. The occurrence of TPs in WWTPs as well as surface waters was investigated. The analysis of groundwater samples as well as of drinking water samples taken from a waterworks utilizing bank filtration and sand filtration allowed for a characterization of the environmental fate of TPs.

Transformation processes in advanced treatment via ozonation.

Chapter 5 reports the removal of antiviral drugs and their transformation products in ozonation. Ozonation rate constants of acyclovir and its biological TP carboxy-acyclovir were determined and oxidation products were identified using high-resolution mass spectrometry and NMR. The toxicity of formed oxidation products was assessed using *Vibrio fischeri*. The occurrence of carboxy-ACV and its main oxidation product in raw and finished drinking water after ozonation and activated carbon treatment was investigated to determine the relevance of the findings.

Final conclusions

Chapter 6 discusses the main outcomes of the studies mentioned above, draws some final conclusions and outlines some further research needs.

1.8 REFERENCES

(1) Halling-Sorensen, B.; Nielsen, S. N.; Lanzky, P. F.; Ingerslev, F.; Lutzhoft, H. C. H.; Jorgensen, S. E., Occurrence, fate and effects of pharmaceutical substances in the environment - A review. Chemosphere 1998, 36 (2), 357-394.

(2) Kummerer, K., Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks. 3rd ed.; Springer Berlin Heidelberg, Germany 2008.

(3) Ternes, T. A.; Joss, A., Human pharmaceuticals, hormones and fragrances: The challenge of micropollutants in urban water management. IWA Publishing: London, UK, 2007.

(4) Daughton, C. G.; Ternes, T. A., Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environmental Health Perspectives 1999, 107, 907-938.

(5) Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L. B.; Buxton, H. T., Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. Environmental Science & Technology 2002, 36 (6), 1202-1211.

(6) Kummerer, K., Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - a review. Chemosphere 2001, 45 (6-7), 957-969.

(7) Ternes, T. A., Occurrence of drugs in German sewage treatment plants and rivers. Water Research 1998, 32 (11), 3245-3260.

(8) Goebel, A.; McArdell, C. S.; Joss, A.; Siegrist, H.; Giger, W., Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. Science of the Total Environment 2007, 372 (2-3), 361-371.

(9) Joss, A.; Zabczynski, S.; Gobel, A.; Hoffmann, B.; Loffler, D.; McArdell, C. S.; Ternes, T. A.; Thomsen, A.; Siegrist, H., Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. Water Research 2006, 40 (8), 1686-1696.

(10) Wick, A.; Fink, G.; Joss, A.; Siegrist, H.; Ternes, T. A., Fate of beta blockers and psycho-active drugs in conventional wastewater treatment. Water Research 2009, 43 (4), 1060-1074.

(11) Horsing, M.; Ledin, A.; Grabic, R.; Fick, J.; Tysklind, M.; Jansen, J. l. C.; Andersen, H. R., Determination of sorption of seventy-five pharmaceuticals in sewage sludge. Water Research 2011, 45 (15), 4470-4482.

(12) Ternes, T. A.; Herrmann, N.; Bonerz, M.; Knacker, T.; Siegrist, H.; Joss, A., A rapid method to measure the solid-water distribution coefficient (K-d) for pharmaceuticals and musk fragrances in sewage sludge. Water Research 2004, 38 (19), 4075-4084.

(13) Wick, A.; Marincas, O.; Moldovan, Z.; Ternes, T. A., Sorption of biocides, triazine and phenylurea herbicides, and UV-filters onto secondary sludge. Water Research 2011, 45 (12), 3638-3652.

(14) Clarke, B. O.; Smith, S. R., Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environment International 2011, 37 (1), 226-247.

(15) Hospido, A.; Carballa, M.; Moreira, M.; Omil, F.; Lema, J. M.; Feijoo, G., Environmental assessment of anaerobically digested sludge reuse in agriculture: Potential impacts of emerging micropollutants. Water Research 2010, 44 (10), 3225-3233.

(16) Jelic, A.; Gros, M.; Ginebreda, A.; Cespedes-Sanchez, R.; Ventura, F.; Petrovic, M.; Barcelo, D., Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. Water Research 2011, 45 (3), 1165-1176.

(17) Kanda, R.; Griffin, P.; James, H. A.; Fothergill, J., Pharmaceutical and personal care products in sewage treatment works. Journal of Environmental Monitoring 2003, 5 (5), 823-830.

(18) Reif, R.; Santos, A.; Judd, S. J.; Lema, J. M.; Omil, F., Occurrence and fate of pharmaceutical and personal care products in a sewage treatment works. Journal of Environmental Monitoring 2011, 13 (1), 137-144.

(19) Buser, H. R.; Poiger, T.; Muller, M. D., Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: Rapid photodegradation in a lake. Environmental Science & Technology 1998, 32 (22), 3449-3456.

(20) Calisto, V.; Domingues, M. R. M.; Esteves, V. I., Photodegradation of psychiatric pharmaceuticals in aquatic environments - Kinetics and photodegradation products. Water research 2011, 45 (18), 6097-106.

(21) Szabo, R. K.; Megyeri, C.; Illes, E.; Gajda-Schrantz, K.; Mazellier, P.; Dombi, A., Phototransformation of ibuprofen and ketoprofen in aqueous solutions. Chemosphere 2011, 84 (11), 1658-1663.

(22) Golet, E. M.; Xifra, I.; Siegrist, H.; Alder, A. C.; Giger, W., Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. Environmental Science & Technology 2003, 37 (15), 3243-3249.

(23) Kahle, M.; Buerge, I. J.; Hauser, A.; Mueller, M. D.; Poiger, T., Azole fungicides: Occurrence and fate in wastewater and surface waters. Environmental Science & Technology 2008, 42 (19), 7193-7200.

(24) Helbling, D. E.; Hollender, J.; Kohler, H.-P. E.; Singer, H.; Fenner, K., High-Throughput Identification of Microbial Transformation Products of Organic Micropollutants. Environmental Science & Technology 2010, 44 (17), 6621-6627.

(25) Schulz, M.; Loffler, D.; Wagner, M.; Ternes, T. A., Transformation of the X-ray contrast medium lopromide in soil and biological wastewater treatment. Environmental Science & Technology 2008, 42 (19), 7207-7217.

(26) Wick, A.; Wagner, M.; Ternes, T. A., Elucidation of the Transformation Pathway of the Opium Alkaloid Codeine in Biological Wastewater Treatment. Environmental Science & Technology 2011, 45 (8), 3374-3385.

(27) Kormos, J. L.; Schulz, M.; Kohler, H. P. E.; Ternes, T. A., Biotransformation of Selected Iodinated X-ray Contrast Media and Characterization of Microbial Transformation Pathways. Environmental Science & Technology 2010, 44 (13), 4998-5007.

(28) Orvos, D. R.; Versteeg, D. J.; Inauen, J.; Capdevielle, M.; Rothenstein, A.; Cunningham, V., Aquatic toxicity of triclosan. Environmental Toxicology and Chemistry 2002, 21 (7), 1338-1349.

(29) Wilson, B. A.; Smith, V. H.; Denoyelles, F.; Larive, C. K., Effects of three pharmaceutical and personal care products on natural freshwater algal assemblages. Environmental Science & Technology 2003, 37 (9), 1713-1719.

(30) LaPara, T. M.; Burch, T. R.; McNamara, P. J.; Tan, D. T.; Yan, M.; Eichmiller, J. J., Tertiary-Treated Municipal Wastewater is a Significant Point Source of Antibiotic Resistance Genes into Duluth-Superior Harbor. Environmental Science & Technology 2011, 45 (22), 9543-9549.

(31) Luczkiewicz, A.; Jankowska, K.; Fudala-Ksiazek, S.; Olanczuk-Neyman, K., Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. Water Research 2010, 44 (17), 5089-5097.

(32) Zhang, Y.; Marrs, C. F.; Simon, C.; Xi, C., Wastewater treatment contributes to selective increase of antibiotic resistance among Acinetobacter spp. Science of the Total Environment 2009, 407 (12), 3702-3706.

(33) Berg, C.; Halldin, K.; Fridolfsson, A. K.; Brandt, I.; Brunstrom, B., The avian egg as a test system for endocrine disrupters: effects of diethylstilbestrol and ethynylestradiol on sex organ development. Science of the Total Environment 1999, 233 (1-3), 57-66.

(34) Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W., Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences of the United States of America 2007, 104 (21), 8897-8901.

(35) Sultan, C.; Balaguer, P.; Terouanne, B.; Georget, V.; Paris, F.; Jeandel, C.; Lumbroso, S.; Nicolas, J. C., Environmental xenoestrogens, antiandrogens and disorders of male sexual differentiation. Mol. Cell. Endocrinol. 2001, 178 (1-2), 99-105.

(36) Xu, Y.; Heilier, J.-F.; Madalinski, G.; Genin, E.; Ezan, E.; Tabet, J.-C.; Junot, C., Evaluation of Accurate Mass and Relative Isotopic Abundance Measurements in

the LTQ-Orbitrap Mass Spectrometer for Further Metabolomics Database Building. Anal. Chem. 2010, 82 (13), 5490-5501.

(37) Fent, K.; Weston, A. A.; Caminada, D., Ecotoxicology of human pharmaceuticals. Aquatic Toxicology 2006, 76 (2), 122-159.

(38) Schwaiger, J.; Ferling, H.; Mallow, U.; Wintermayr, H.; Negele, R. D., Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part 1: histopathological alterations and bioaccumulation in rainbow trout. Aquatic Toxicology 2004, 68 (2), 141-150.

(39) Oaks, J. L.; Gilbert, M.; Virani, M. Z.; Watson, R. T.; Meteyer, C. U.; Rideout, B. A.; Shivaprasad, H. L.; Ahmed, S.; Chaudhry, M. J. I.; Arshad, M., et al., Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 2004, 427 (6975), 630-633.

(40) Celiz, M. D.; Tso, J.; Aga, D. S., Pharmaceutical Metabolites in the Environment: Analytical Challenges and Ecological Risks. Environmental Toxicology and Chemistry 2009, 28 (12), 2473-2484.

(41) van Zelm, R.; Huijbregts, M. A. J.; van de Meent, D., Transformation Products in the Life Cycle Impact Assessment of Chemicals. Environmental Science & Technology 2010, 44 (3), 1004-1009.

(42) Escher, B. I.; Fenner, K., Recent Advances in Environmental Risk Assessment of Transformation Products. Environmental Science & Technology 2011, 45 (9), 3835-3847.

(43) Bond, T.; Huang, J.; Templeton, M. R.; Graham, N., Occurrence and control of nitrogenous disinfection by-products in drinking water - A review. Water Research 2011, 45 (15), 4341-4354.

(44) Krasner, S. W., The formation and control of emerging disinfection by-products of health concern. Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences 2009, 367 (1904), 4077-4095.

(45) Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; DeMarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: A review and roadmap for research. Mutation Research-Reviews in Mutation Research 2007, 636 (1-3), 178-242.

(46) Le Roux, J.; Gallard, H.; Croue, J.-P., Chloramination of nitrogenous contaminants (pharmaceuticals and pesticides): NDMA and halogenated DBPs formation. Water Research 2011, 45 (10), 3164-3174.

(47) Shen, R.; Andrews, S. A., NDMA formation kinetics from three pharmaceuticals in four water matrices. Water Research 2011, 45 (17), 5687-5694.

(48) Schmidt, C. K.; Brauch, H.-J., N,N-dimethosulfamide as precursor for N-nitrosodimethylamine (NDMA) formation upon ozonation and its fate during drinking water treatment. Environ. Sci. Technol. 2008, 42 (17), 6340-6346.

(49) Eichhorn, P.; Ferguson, P. L.; Perez, S.; Aga, D. S., Application of ion trap-MS with QqTOF-MS in the identification H/D exchange and of microbial degradates of trimethoprim in nitrifying activated sludge. Anal. Chem. 2005, 77 (13), 4176-4184.

(50) Helbling, D. E.; Hollender, J.; Kohler, H.-P. E.; Fenner, K., Structure-Based Interpretation of Biotransformation Pathways of Amide-Containing Compounds in Sludge-Seeded Bioreactors. Environmental Science & Technology 2010, 44 (17), 6628-6635.

(51) Kern, S.; Fenner, K.; Singer, H. P.; Schwarzenbach, R. P.; Hollender, J., Identification of Transformation Products of Organic Contaminants in Natural Waters by Computer-Aided Prediction and High-Resolution Mass Spectrometry. Environmental Science & Technology 2009, 43 (18), 7039-7046.

(52) Kosjek, T.; Heath, E.; Perez, S.; Petrovic, M.; Barcelo, D., Metabolism studies of diclofenac and clofibric acid in activated sludge bioreactors using liquid chromatography with quadrupole - time-of-flight mass spectrometry. Journal of Hydrology 2009, 372 (1-4), 109-117.

(53) Quintana, J. B.; Rodil, R.; Lopez-Mahia, P.; Muniategui-Lorenzo, S.; Prada-Rodriguez, D., Investigating the chlorination of acidic pharmaceuticals and by-product formation aided by an experimental design methodology. Water Research 2010, 44 (1), 243-255.

(54) Radjenovic, J.; Pereza, S.; Petrovic, M.; Barcelo, D., Identification and structural characterization of biodegradation products of atenolol and glibenclamide by liquid chromatography coupled to hybrid quadrupole time-of-flight and quadrupole ion trap mass spectrometry. J. Chromatogr. A 2008, 1210 (2), 142-153.

(55) Trautwein, C.; Kummerer, K.; Metzger, J. W., Aerobic biodegradability of the calcium channel antagonist verapamil and identification of a microbial dead-end transformation product studied by LC-MS/MS. Chemosphere 2008, 72 (3), 442-450.

(56) Zwiener, C.; Seeger, S.; Glauner, T.; Frimmel, F. H., Metabolites from the biodegradation of pharmaceutical residues of ibuprofen in biofilm reactors and batch experiments. Anal. Bioanal. Chem. 2002, 372 (4), 569-575.

(57) Hollender, J.; Zimmermann, S. G.; Koepke, S.; Krauss, M.; McArdell, C. S.; Ort, C.; Singer, H.; von Gunten, U.; Siegrist, H., Elimination of Organic Micropollutants in a Municipal Wastewater Treatment Plant Upgraded with a Full-Scale Post-Ozonation Followed by Sand Filtration. Environmental Science & Technology 2009, 43 (20), 7862-7869.

(58) Yang, X.; Flowers, R. C.; Weinberg, H. S.; Singer, P. C., Occurrence and removal of pharmaceuticals and personal care products (PPCPs) in an advanced wastewater reclamation plant. Water Research 2011, 45 (16), 5218-5228.

(59) Reungoat, J.; Macova, M.; Escher, B. I.; Carswell, S.; Mueller, J. F.; Keller, J., Removal of micropollutants and reduction of biological activity in a full scale reclamation plant using ozonation and activated carbon filtration. Water Research 2010, 44 (2), 625-637.

(60) Ruel, S. M.; Choubert, J. M.; Esperanza, M.; Miege, C.; Navalon Madrigal, P.; Budzinski, H.; Le Menach, K.; Lazarova, V.; Coquery, M., On-site evaluation of the removal of 100 micro-pollutants through advanced wastewater treatment processes for reuse applications. Water Science and Technology 2011, 63 (11), 2486-2497.

(61) Nowotny, N.; Epp, B.; von Sonntag, C.; Fahlenkamp, H., Quantification and modeling of the elimination behavior of ecologically problematic wastewater micropollutants by adsorption on powdered and granulated activated carbon. Environmental Science & Technology 2007, 41 (6), 2050-2055.

(62) von Gunten, U., Ozonation of drinking water: Part I. Oxidation kinetics and product formation. Water Research 2003, 37 (7), 1443-1467.

(63) Dodd, M. C.; Buffle, M. O.; Von Gunten, U., Oxidation of antibacterial molecules by aqueous ozone: Moiety-specific reaction kinetics and application to ozone-based wastewater treatment. Environmental Science & Technology 2006, 40 (6), 1969-1977.

(64) Huber, M. M.; Gobel, A.; Joss, A.; Hermann, N.; Loffler, D.; McArdell, C. S.; Ried, A.; Siegrist, H.; Ternes, T. A.; von Gunten, U., Oxidation of pharmaceuticals during ozonation of municipal wastewater effluents: A pilot study. Environmental Science & Technology 2005, 39 (11), 4290-4299.

(65) Huber, M. M.; Ternes, T. A.; von Gunten, U., Removal of estrogenic activity and formation of oxidation products during ozonation of 17 alpha-ethinylestradiol. Environmental Science & Technology 2004, 38 (19), 5177-5186.

(66) Ternes, T. A.; Stuber, J.; Herrmann, N.; McDowell, D.; Ried, A.; Kampmann, M.; Teiser, B., Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? Water Research 2003, 37 (8), 1976-1982.

(67) Hoigne, J.; Bader, H., Ozonation of Water - Role of Hydroxyl Radicals as Oxidizing Intermediates. Science 1975, 190 (4216), 782-784.

(68) von Gunten, U., The basics of oxidants in water treatment. Part B: ozone reactions. Water Science and Technology 2007, 55 (12), 25-29.

(69) Buffle, M. O.; Von Gunten, U., Phenols and amine induced HO center dot generation during the initial phase of natural water ozonation. Environmental Science & Technology 2006, 40 (9), 3057-3063.

(70) Elovitz, M. S.; von Gunten, U.; Kaiser, H. P., Hydroxyl radical/ozone ratios during ozonation processes. II. The effect of temperature, pH, alkalinity, and DOM properties. Ozone-Science & Engineering 2000, 22 (2), 123-150.

(71) Staehelin, J.; Hoigne, J., Decomposition of Ozone in Water in the Presence of Organic Solutes Acting as Promoters and Inhibitors of Radical Chain Reactions. Environmental Science & Technology 1985, 19 (12), 1206-1213.

(72) Haag, W. R.; Yao, C. C. D., Rate Constants for Reaction of Hydroxyl Radicals with Several Drinking-Water Contaminants. Environmental Science & Technology 1992, 26 (5), 1005-1013.

(73) Yao, C. C. D.; Haag, W. R., Rate Constants for Direct Reactions of Ozone with Several Drinking-Water Contaminants. Water Research 1991, 25 (7), 761-773.

(74) Hoigne, J.; Bader, H., Rate Constants of Reactions of Ozone with Organic and Inorganic-Compounds in Water .1. Non-Dissociating Organic-Compounds. Water Research 1983, 17 (2), 173-183.

(75) Hoigne, J.; Bader, H., Rate Constants of Reactions of Ozone with Organic and Inorganic-Compounds in Water .2. Dissociating Organic-Compounds. Water Research 1983, 17 (2), 185-194.

(76) Teixeira, M. R.; Rosa, S. M.; Sousa, V., Natural Organic Matter and Disinfection By-products Formation Potential in Water Treatment. Water Resources Management 2011, 25 (12), 3005-3015.

(77) von Gunten, U., Ozonation of drinking water: Part II. Disinfection and by-product formation in presence of bromide, iodide or chlorine. Water Research 2003, 37 (7), 1469-1487.

(78) von Gunten, U.; Driedger, A.; Gallard, H.; Salhi, E., By-products formation during drinking water disinfection: A tool to assess disinfection efficiency? Water Research 2001, 35 (8), 2095-2099.

(79) Zimmermann, S. G.; Wittenwiler, M.; Hollender, J.; Krauss, M.; Ort, C.; Siegrist, H.; von Gunten, U., Kinetic assessment and modeling of an ozonation step for full-scale municipal wastewater treatment: Micropollutant oxidation, by-product formation and disinfection. Water Research 2011, 45 (2), 605-617.

(80) Alvares, A. B. C.; Diaper, C.; Parsons, S. A., Partial oxidation by ozone to remove recalcitrance from wastewaters - A review. Environmental Technology 2001, 22 (4), 409-427.

(81) Bundschuh, M.; Schulz, R., Ozonation of secondary treated wastewater reduces ecotoxicity to Gammarus fossarum (Crustacea; Amphipoda): Are loads of (micro)pollutants responsible? Water Research 2011, 45 (13), 3999-4007.

(82) Li, L. S.; Zhu, W. P.; Zhang, P. Y.; Zhang, Z. L.; Wu, H. H.; Han, W. Y., Comparison of AC/O-3-BAC and O-3-BAC processes for removing organic pollutants in secondary effluent. Chemosphere 2006, 62 (9), 1514-1522.

(83) Misik, M.; Knasmueller, S.; Ferk, F.; Cichna-Markl, M.; Grummt, T.; Schaar, H.; Kreuzinger, N., Impact of ozonation on the genotoxic activity of tertiary treated municipal wastewater. Water Research 2011, 45 (12), 3681-3691.

(84) Saroj, D. P.; Kumar, A.; Bose, P.; Tare, V., Enhancement in mineralization of some natural refractory organic compounds by ozonation-aerobic biodegradation. Journal of Chemical Technology and Biotechnology 2006, 81 (2), 115-127.

(85) Guelli Ulson de Souza, S. M. d. A.; Santos Bonilla, K. A.; Ulson de Souza, A. A., Removal of COD and color from hydrolyzed textile azo dye by combined ozonation and biological treatment. Journal of Hazardous Materials 2010, 179 (1-3), 35-42.

(86) Sangave, P. C.; Gogate, P. R.; Pandit, A. B., Combination of ozonation with conventional aerobic oxidation for distillery wastewater treatment. Chemosphere 2007, 68 (1), 32-41.

(87) Kormos, J. L.; Schulz, M.; Ternes, T. A., Occurrence of Iodinated X-ray Contrast Media and Their Biotransformation Products in the Urban Water Cycle. Environmental science & technology 2011, 45 (20), 8723-32.

(88) Duirk, S. E.; Lindell, C.; Cornelison, C. C.; Kormos, J.; Ternes, T. A.; Attene-Ramos, M.; Osiol, J.; Wagner, E. D.; Plewa, M. J.; Richardson, S. D., Formation of Toxic Iodinated Disinfection By-Products from Compounds Used in Medical Imaging. Environmental Science & Technology 2011, 45 (16), 6845-6854.

(89) Field, J. A.; Sierra-Alvarez, R., Microbial degradation of chlorinated benzenes. Biodegradation 2008, 19 (4), 463-480.

(90) Haggblom, M. M.; Knight, V. K.; Kerkhof, L. J., Anaerobic decomposition of halogenated aromatic compounds. Environmental Pollution 2000, 107 (2), 199-207.

(91) Heimann, A. C.; Friis, A. K.; Jakobsen, R., Effects of sulfate on anaerobic chloroethene degradation by an enriched culture under transient and steady-state hydrogen supply. Water Research 2005, 39 (15), 3579-3586.

(92) Shen, H.; Sewell, G. W., Reductive biotransformation of tetrachloroethene to ethene during anaerobic degradation of toluene: Experimental evidence and kinetics. Environmental Science & Technology 2005, 39 (23), 9286-9294.

(93) Aslan, S.; Miller, L.; Dahab, M., Ammonium oxidation via nitrite accumulation under limited oxygen concentration in sequencing batch reactors. Bioresource Technology 2009, 100 (2), 659-664.

(94) Ferguson, J. F., Anaerobic and Aerobic Treatment for Aox Removal. Water Science and Technology 1994, 29 (5-6), 149-162.

(95) Goncharuk, V. V.; Bagrii, V. A.; Mel'nik, L. A.; Chebotareva, R. D.; Bashtan, S. Y., The use of redox potential in water treatment processes. Journal of Water Chemistry and Technology 2010, 32 (1), 1-9.

(96) Yoo, K.; Ahn, K. H.; Lee, H. J.; Lee, K. H.; Kwak, Y. J.; Song, K. G., Nitrogen removal from synthetic wastewater by simultaneous nitrification and

denitrification (SND) via nitrite in an intermittently-aerated reactor. Water Research 1999, 33 (1), 145-154.

(97) Garibay-Orijel, C.; Rios-Leal, E.; Garcia-Mena, J.; Poggi-Varaldo, H. M., 2,4,6-Trichlorophenol and phenol removal in methanogenic and partially-aerated methanogenic conditions in a fluidized bed bioreactor. Journal of Chemical Technology and Biotechnology 2005, 80 (10), 1180-1187.

(98) Popat, S. C.; Deshusses, M. A., Kinetics and Inhibition of Reductive Dechlorination of Trichloroethene, cis-1,2-Dichloroethene and Vinyl Chloride in a Continuously Fed Anaerobic Biofilm Reactor. Environmental Science & Technology 2011, 45 (4), 1569-1578.

(99) Quintero, J. C.; Moreira, M. T.; Lema, J. M.; Feijoo, G., An anaerobic bioreactor allows the efficient degradation of HCH isomers in soil slurry. Chemosphere 2006, 63 (6), 1005-1013.

(100) Cakir, F. Y.; Stenstrom, M. K., Greenhouse gas production: A comparison between aerobic and anaerobic wastewater treatment technology. Water Research 2005, 39 (17), 4197-4203.

(101) Mannucci, A.; Munz, G.; Mori, G.; Lubello, C., Anaerobic treatment of vegetable tannery wastewaters: A review. Desalination 2011, 264 (1-2), 1-8.

(102) Rodriguez-Martinez, J.; Garza-Garcia, Y.; Aguilera-Carbo, A.; Martinez-Amador, S. Y.; Sosa-Santillan, G. J., Influence of nitrate and sulfate on the anaerobic treatment of pharmaceutical wastewater. Eng. Life Sci. 2005, 5 (6), 568-573.

(103) Yang, M. I.; Edwards, E. A.; Allen, D. G., Anaerobic treatability and biogas production potential of selected in-mill streams. Water Science and Technology 2010, 62 (10), 2427-2434.

(104) Basile, T.; Petrella, A.; Petrella, M.; Boghetich, G.; Petruzzelli, V.; Colasuonno, S.; Petruzzelli, D., Review of Endocrine-Disrupting-Compound Removal Technologies in Water and Wastewater Treatment Plants: An EU Perspective. Industrial & Engineering Chemistry Research 2011, 50 (14), 8389-8401.

(105) Magureanu, M.; Piroi, D.; Mandache, N. B.; David, V.; Medvedovici, A.; Bradu, C.; Parvulescu, V. I., Degradation of antibiotics in water by non-thermal plasma treatment. Water Research 2011, 45 (11), 3407-3416.

(106) Brillas, E.; Sires, I.; Oturan, M. A., Electro-Fenton Process and Related Electrochemical Technologies Based on Fenton's Reaction Chemistry. Chemical Reviews 2009, 109 (12), 6570-6631.

(107) Chong, M. N.; Jin, B.; Chow, C. W. K.; Saint, C., Recent developments in photocatalytic water treatment technology: A review. Water Research 2010, 44 (10), 2997-3027.

(108) Deegan, A. M.; Shaik, B.; Nolan, K.; Urell, K.; Oelgemoeller, M.; Tobin, J.; Morrissey, A., Treatment options for wastewater effluents from pharmaceutical

companies. International Journal of Environmental Science and Technology 2011, 8 (3), 649-666.

(109) Fatta-Kassinos, D.; Meric, S.; Nikolaou, A., Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. Anal. Bioanal. Chem. 2011, 399 (1), 251-275.

(110) Petrovic, M.; Lopez De Alda, M. J.; Diaz-Cruz, S.; Postigo, C.; Radjenovic, J.; Gros, M.; Barcelo, D., Fate and removal of pharmaceuticals and illicit drugs in conventional and membrane bioreactor wastewater treatment plants and by riverbank filtration. Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences 2009, 367 (1904), 3979-4003.

(111) Bagastyo, A. Y.; Radjenovic, J.; Mu, Y.; Rozendal, R. A.; Batstone, D. J.; Rabaey, K., Electrochemical oxidation of reverse osmosis concentrate on mixed metal oxide (MMO) titanium coated electrodes. Water Research 2011, 45 (16), 4951-4959.

(112) Bolong, N.; Ismail, A. F.; Salim, M. R.; Matsuura, T., A review of the effects of emerging contaminants in wastewater and options for their removal. Desalination 2009, 239 (1-3), 229-246.

(113) Joss, A.; Baenninger, C.; Foa, P.; Koepke, S.; Krauss, M.; McArdell, C. S.; Rottermann, K.; Wei, Y.; Zapata, A.; Siegrist, H., Water reuse: >90% water yield in MBR/RO through concentrate recycling and CO(2) addition as scaling control. Water research 2011, 45 (18), 6141-51.

(114) Klavarioti, M.; Mantzavinos, D.; Kassinos, D., Removal of residual pharmaceuticals from aqueous systems by advanced oxidation processes. Environment International 2009, 35 (2), 402-417.

(115) Kent, F. C.; Farahbakhsh, K.; Mahendran, B.; Jaklewicz, M.; Liss, S. N.; Zhou, H., Water reclamation using reverse osmosis: Analysis of fouling propagation given tertiary membrane filtration and MBR pretreatments. Journal of Membrane Science 2011, 382 (1-2), 328-338.

(116) Oller, I.; Malato, S.; Sanchez-Perez, J. A., Combination of Advanced Oxidation Processes and biological treatments for wastewater decontamination-A review. Science of the Total Environment 2011, 409 (20), 4141-4166.

(117) Beier, S.; Cramer, C.; Koester, S.; Mauer, C.; Palmowski, L.; Schroeder, H. F.; Pinnekamp, J., Full scale membrane bioreactor treatment of hospital wastewater as forerunner for hot-spot wastewater treatment solutions in high density urban areas. Water Science and Technology 2011, 63 (1), 66-71.

(118) Lienert, J.; Buerki, T.; Escher, B. I., Reducing micropollutants with source control: substance flow analysis of 212 pharmaceuticals in faeces and urine. Water Science and Technology 2007, 56 (5), 87-96.

(119) Lienert, J.; Koller, M.; Konrad, J.; McArdell, C. S.; Schuwirth, N., Multiple-Criteria Decision Analysis Reveals High Stakeholder Preference to Remove

Pharmaceuticals from Hospital Wastewater. Environmental Science & Technology 2011, 45 (9), 3848-3857.

(120) Lienert, J.; Larsen, T. A., Soft paths in wastewater management - The pros and cons of urine source separation. Gaia-Ecological Perspectives for Science and Society 2007, 16 (4), 280-288.

(121) Nurmi, J.; Pellinen, J., Multiresidue method for the analysis of emerging contaminants in wastewater by ultra performance liquid chromatography-time-of-flight mass spectrometry. J. Chromatogr. A 2011, 1218 (38), 6712-6719.

(122) Ollers, S.; Singer, H. P.; Fassler, P.; Muller, S. R., Simultaneous quantification of neutral and acidic pharmaceuticals and pesticides at the low-ng/1 level in surface and waste water. J. Chromatogr. A 2001, 911 (2), 225-234.

(123) Gomez, M. J.; Gomez-Ramos, M. M.; Malato, O.; Mezcua, M.; Fernandez-Alba, A. R., Rapid automated screening, identification and quantification of organic micro-contaminants and their main transformation products in wastewater and river waters using liquid chromatography-quadrupole-time-of-flight mass spectrometry with an accurate-mass database. J. Chromatogr. A 2010, 1217 (45), 7038-7054.

(124) Pitarch, E.; Portoles, T.; Marin, J. M.; Ibanez, M.; Albarran, F.; Hernandez, F., Analytical strategy based on the use of liquid chromatography and gas chromatography with triple-quadrupole and time-of-flight MS analyzers for investigating organic contaminants in wastewater. Anal. Bioanal. Chem. 2010, 397 (7), 2763-2776.

(125) Dunn, W. B.; Bailey, N. J. C.; Johnson, H. E., Measuring the metabolome: current analytical technologies. Analyst 2005, 130 (5), 606-625.

(126) Giavalisco, P.; Hummel, J.; Lisec, J.; Inostroza, A. C.; Catchpole, G.; Willmitzer, L., High-Resolution Direct Infusion-Based Mass Spectrometry in Combination with Whole (13)C Metabolome Isotope Labeling Allows Unambiguous Assignment of Chemical Sum Formulas. Anal. Chem. 2008, 80 (24), 9417-9425.

(127) Han, J.; Danell, R. M.; Patel, J. R.; Gumerov, D. R.; Scarlett, C. O.; Speir, J. P.; Parker, C. E.; Rusyn, I.; Zeisel, S.; Borchers, C. H., Towards high-throughput metabolomics using ultrahigh-field Fourier transform ion cyclotron resonance mass spectrometry. Metabolomics 2008, 4 (2), 128-140.

(128) Kind, T.; Fiehn, O., Metabolomic database annotations via query of elemental compositions: Mass accuracy is insufficient even at less than 1 ppm. Bmc Bioinformatics 2006, 7.

(129) Krauss, M.; Singer, H.; Hollender, J., LC-high resolution MS in environmental analysis: from target screening to the identification of unknowns. Anal. Bioanal. Chem. 2010, 397 (3), 943-951.

(130) Muller, A.; Schulz, W.; Ruck, W. K. L.; Weber, W. H., A new approach to data evaluation in the non-target screening of organic trace substances in water analysis. Chemosphere 2011, 85 (8), 1211-9.

(132) Pluskal, T.; Castillo, S.; Villar-Briones, A.; Oresic, M., MZmine 2: Modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. Bmc Bioinformatics 2010, 11.

(132) Takahashi, H.; Morimoto, T.; Ogasawara, N.; Kanaya, S., AMDORAP: Non-targeted metabolic profiling based on high-resolution LC-MS. Bmc Bioinformatics 2011, 12.

(133) Wei, X.; Sun, W.; Shi, X.; Koo, I.; Wang, B.; Zhang, J.; Yin, X.; Tang, Y.; Bogdanov, B.; Kim, S., et al., MetSign: A Computational Platform for High-Resolution Mass Spectrometry-Based Metabolomics. Anal. Chem. 2011, 83 (20), 7668-7675.

(134) Han, J.; Datla, R.; Chan, S.; Borchers, C. H., Mass spectrometry-based technologies for high-throughput metabolomics. Bioanalysis 2009, 1 (9), 1665-1684.

(135) Hildebrandt, C.; Wolf, S.; Neumann, S., Database supported candidate search for metabolite identification. Journal of integrative bioinformatics 2011, 8 (2), 157.

(136) Neumann, S.; Boecker, S., Computational mass spectrometry for metabolomics: Identification of metabolites and small molecules. Anal. Bioanal. Chem. 2010, 398 (7-8), 2779-2788.

(137) Wolf, S.; Schmidt, S.; Mueller-Hannemann, M.; Neumann, S., In silico fragmentation for computer assisted identification of metabolite mass spectra. Bmc Bioinformatics 2010, 11.

(138) Zhang, H.; Grubb, M.; Wu, W.; Josephs, J.; Humphreys, W. G., Algorithm for Thorough Background Subtraction of High-Resolution LC/MS Data: Application to Obtain Clean Product Ion Spectra from Nonselective Collision-Induced Dissociation Experiments. Anal. Chem. 2009, 81 (7), 2695-2700.

(139) Borland, L.; Brickhouse, M.; Thomas, T.; Fountain, A. W., III, Review of chemical signature databases. Anal. Bioanal. Chem. 2010, 397 (3), 1019-1028.

(140) Stein, S. E., Chemical Substructure Identification by Mass-Spectral Library Searching. Journal of the American Society for Mass Spectrometry 1995, 6 (8), 644-655.

(141) Alder, L.; Greulich, K.; Kempe, G.; Vieth, B., Residue analysis of 500 high priority pesticides: Better by GC-MS or LC-MS/MS? Mass Spectrometry Reviews 2006, 25 (6), 838-865.

(142) Liu, H.-C.; Liu, R. H.; Ho, H.-O.; Lin, D.-L., Development of an Information-Rich LC-MS/MS Database for the Analysis of Drugs in Postmortem Specimens. Anal. Chem. 2009, 81 (21), 9002-9011.

(143) Niessen, W. M. A., Group-specific fragmentation of pesticides and related compounds in liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 2010, 1217 (25), 4061-4070.

(144) Gomez-Ramos, M. d. M.; Perez-Parada, A.; Garcia-Reyes, J. F.; Fernandez-Alba, A. R.; Aguera, A., Use of an accurate-mass database for the systematic identification of transformation products of organic contaminants in wastewater effluents. Journal of chromatography. A 2011, 1218 (44), 8002-12.

(145) Gao, J.; Ellis, L. B. M.; Wackett, L. P., The University of Minnesota Pathway Prediction System: multi-level prediction and visualization. Nucleic Acids Research 2011, 39, W406-W411.

(146) Hou, B. K.; Wackett, L. P.; Ellis, L. B. M., Microbial pathway prediction: A functional group approach. J. Chem. Inf. Comput. Sci. 2003, 43 (3), 1051-1057.

(147) Jaworska, J.; Dimitrov, S.; Nikolova, N.; Mekenyan, O., Probabilistic assessment of biodegradability based on metabolic pathways: Catabol system. SAR QSAR Environ. Res. 2002, 13 (2), 307-323.

(148) Cardona, S.; Suarez, E., Biodegradation pathway prediction of POPs (Persistent organic pollutants) and biobarrier treatment. Dyna-Colombia 2010, 77 (163), 115-123.

(149) Schenker, U.; Scheringer, M.; Hungerbuhler, K., Including degradation products of persistent organic pollutants in a global multi-media box model. Environ. Sci. Pollut. Res. 2007, 14 (3), 145-152.

(150) Wicker, J.; Fenner, K.; Ellis, L.; Wackett, L.; Kramer, S., Predicting biodegradation products and pathways: a hybrid knowledge- and machine learning-based approach. Bioinformatics 2010, 26 (6), 814-821.

(151) Brack, W., Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures? Anal. Bioanal. Chem. 2003, 377 (3), 397-407.

(152) Hecker, M.; Hollert, H., Effect-directed analysis (EDA) in aquatic ecotoxicology: state of the art and future challenges. Environ. Sci. Pollut. Res. 2009, 16 (6), 607-613.

(153) Choma, I. M.; Grzelak, E. M., Bioautography detection in thin-layer chromatography. J. Chromatogr. A 2011, 1218 (19), 2684-2691.

(154) Devier, M.-H.; Mazellier, P.; Ait-Aissa, S.; Budzinski, H., New challenges in environmental analytical chemistry: Identification of toxic compounds in complex mixtures. Comptes Rendus Chimie 2011, 14 (7-8), 766-779.

(155) Weiss, J. M.; Simon, E.; Stroomberg, G. J.; de Boer, R.; de Boer, J.; van der Linden, S. C.; Leonards, P. E. G.; Lamoree, M. H., Identification strategy for unknown pollutants using high-resolution mass spectrometry: Androgen-disrupting compounds identified through effect-directed analysis. Anal. Bioanal. Chem. 2011, 400 (9), 3141-3149.

(156) Schulze, T.; Weiss, S.; Schymanski, E.; von der Ohe, P. C.; Schmitt-Jansen, M.; Altenburger, R.; Streck, G.; Brack, W., Identification of a phytotoxic photo-transformation product of diclofenac using effect-directed analysis. Environmental Pollution 2010, 158 (5), 1461-1466.

(157) Eberz, G.; Rast, H. G.; Burger, K.; Kreiss, W.; Weisemann, C., Bioactivity screening by chromatography-bioluminescence coupling. Chromatographia 1996, 43 (1-2), 5-9.

(158) Schulz, W.; Seitz, W.; Weiss, S. C.; Weber, W. H.; Bohm, M.; Flottmann, D., Use of Vibrio fischeri for Screening for Bioactivity in Water Analysis. JPC-J. Planar Chromatogr.-Mod. TLC 2008, 21 (6), 427-430.

(159) Kool, J.; Giera, M.; Irth, H.; Niessen, W. M. A., Advances in mass spectrometry-based post-column bioaffinity profiling of mixtures. Anal. Bioanal. Chem. 2011, 399 (8), 2655-2668.

(160) Walz, I.; Schwack, W., Multienzyme inhibition assay for residue analysis of insecticidal organophosphates and carbamates. Journal of Agricultural and Food Chemistry 2007, 55 (26), 10563-10571.

(161) Hogenboom, A. C.; de Boer, A. R.; Derks, R. J. E.; Irth, H., Continuous-flow, on-line monitoring of biospecific interactions using electrospray mass spectrometry. Anal. Chem. 2001, 73 (16), 3816-3823.

(162) Akkad, R.; Schwack, W., Multi-enzyme inhibition assay for the detection of insecticidal organophosphates and carbamates by high-performance thin-layer chromatography applied to determine enzyme inhibition factors and residues in juice and water samples. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2010, 878 (17-18), 1337-1345.

(163) Weins, C.; Jork, H., Toxicological evaluation of harmful substances by in situ enzymatic and biological detection in high-performance thin-layer chromatography. J. Chromatogr. A 1996, 750 (1-2), 403-407.

(164) Cervantes, F. J.; Gutierrez, C. H.; Lopez, K. Y.; Estrada-Alvarado, M. I.; Meza-Escalante, E. R.; Texier, A.-C.; Cuervo, F.; Gomez, J., Contribution of quinone-reducing microorganisms to the anaerobic biodegradation of organic compounds under different redox conditions. Biodegradation 2008, 19 (2), 235-246.

(165) Liu, Y.-S.; Ying, G.-G.; Shareef, A.; Kookana, R. S., Biodegradation of three selected benzotriazoles under aerobic and anaerobic conditions. Water Research 2011, 45 (16), 5005-5014.

(166) van der Zaan, B.; de Weert, J.; Rijnaarts, H.; de Vos, W. M.; Smidt, H.; Gerritse, J., Degradation of 1,2-dichloroethane by microbial communities from river sediment at various redox conditions. Water Research 2009, 43 (13), 3207-3216.

(167) Ginestet, P.; Audic, J. M.; Urbain, V.; Block, J. C., Estimation of nitrifying bacterial activities by measuring oxygen uptake in the presence of the metabolic inhibitors allylthiourea and azide. Applied and Environmental Microbiology 1998, 64 (6), 2266-2268.

(168) Munz, G.; Mori, G.; Vannini, C.; Lubello, C., Kinetic parameters and inhibition response of ammonia- and nitrite-oxidizing bacteria in membrane bioreactors and conventional activated sludge processes. Environmental Technology 2010, 31 (14), 1557-1564.

(169) Abe, Y.; Aravena, R.; Zopfi, J.; Parker, B.; Hunkeler, D., Evaluating the fate of chlorinated ethenes in streambed sediments by combining stable isotope, geochemical and microbial methods. Journal of Contaminant Hydrology 2009, 107 (1-2), 10-21.

(170) Youngster, L. K. G.; Rosell, M.; Richnow, H. H.; Haggblom, M. M., Assessment of MTBE biodegradation pathways by two-dimensional isotope analysis in mixed bacterial consortia under different redox conditions. Applied Microbiology and Biotechnology 2010, 88 (1), 309-317.

(171) Liu, J.; Wang, N.; Buck, R. C.; Wolstenholme, B. W.; Folsom, P. W.; Sulecki, L. M.; Bellin, C. A., Aerobic biodegradation of [(14)C] 6:2 fluorotelomer alcohol in a flow-through soil incubation system. Chemosphere 2010, 80 (7), 716-723.

(172) Loffler, D.; Rombke, J.; Meller, M.; Ternes, T. A., Environmental fate of pharmaceuticals in water/sediment systems. Environmental Science & Technology 2005, 39 (14), 5209-5218.

(173) Prasse, C.; Loffler, D.; Ternes, T. A., Environmental fate of the anthelmintic ivermectin in an aerobic sediment/water system. Chemosphere 2009, 77 (10), 1321-1325.

(174) Snyder, E. H.; O'Connor, G. A.; McAvoy, D. C., Fate of (14)C-triclocarban in biosolids-amended soils. Science of the Total Environment 2010, 408 (13), 2726-2732.

(175) De Clercq, E., A 40-Year Journey in Search of Selective Antiviral Chemotherapy. In Annual Review of Pharmacology and Toxicology, Vol 51, 2011, 2011; Vol. 51, pp 1-24.

(176) De Clercq, E., Three decades of antiviral drugs. Nat. Rev. Drug Discov. 2007, 6 (12), 941-941.

(177) De Clercq, E., Strategies in the design of antiviral drugs. Nat. Rev. Drug Discov. 2002, 1 (1), 13-25.

(178) Allen, M. J.; Wilson, W. H., Aquatic virus diversity accessed through omic techniques: A route map to function. Current Opinion in Microbiology 2008, 11 (3), 226-232.

(179) Winget, D. M.; Helton, R. R.; Williamson, K. E.; Bench, S. R.; Williamson, S. J.; Wommack, K. E., Repeating patterns of virioplankton production within an

estuarine ecosystem. Proceedings of the National Academy of Sciences of the United States of America 2011, 108 (28), 11506-11511.

(180) Berdjeb, L.; Jacquet, S., The viriosphere: What place in the functioning and evolution of aquatic ecosystems (part 1)? Virologie 2009, 13 (3), 133-143.

(181) Wommack, K. E.; Colwell, R. R., Virioplankton: Viruses in aquatic ecosystems. Microbiology and Molecular Biology Reviews 2000, 64 (1), 69-+.

(182) Jarhult, J. D.; Muradrasoli, S.; Wahlgren, J.; Soderstrom, H.; Orozovic, G.; Gunnarsson, G.; Brojer, C.; Latorre-Margalef, N.; Fick, J.; Grabic, R., et al., Environmental levels of the antiviral oseltamivir induce development of resistance mutation H274Y in influenza A/H1N1 virus in mallards. PloS one 2011, 6 (9), e24742.

(183) Singer, A. C.; Howard, B. M.; Johnson, A. C.; Knowles, C. J.; Jackman, S.; Accinelli, C.; Caracciolo, A. B.; Bernard, I.; Bird, S.; Boucard, T., et al., Meeting Report: Risk Assessment of Tamiflu Use Under Pandemic Conditions. Environmental Health Perspectives 2008, 116 (11), 1563-1567.

(184) Olsen, B.; Munster, V. J.; Wallensten, A.; Waldenstrom, J.; Osterhaus, A.; Fouchier, R. A. M., Global patterns of influenza A virus in wild birds. Science 2006, 312 (5772), 384-388.

(185) Singer, A. C.; Colizza, V.; Schmitt, H.; Andrews, J.; Balcan, D.; Huang, W. E.; Keller, V. D. J.; Vespignani, A.; Williams, R. J., Assessing the Ecotoxicologic Hazards of a Pandemic Influenza Medical Response. Environmental Health Perspectives 2011, 119 (8), 1084-1090.

(186) Ellis, J. B., Antiviral pandemic risk assessment for urban receiving waters. Water Science and Technology 2010, 61 (4), 879-884.

(187) Escher, B. I.; Bramaz, N.; Lienert, J.; Neuwoehner, J.; Straub, J. O., Mixture toxicity of the antiviral drug Tamiflu (R) (oseltamivir ethylester) and its active metabolite oseltamivir acid. Aquatic Toxicology 2010, 96 (3), 194-202.

(188) Hutchinson, T. H.; Beesley, A.; Frickers, P. E.; Readman, J. W.; Shaw, J. P.; Straub, J. O., Extending the environmental risk assessment for oseltamivir (Tamiflu (R)) under pandemic use conditions to the coastal marine compartment. Environment International 2009, 35 (6), 931-936.

(188) Straub, J. O., An environmental risk assessment for oseltamivir (Tamiflu (R)) for sewage works and surface waters under seasonal-influenza- and pandemic-use conditions. Ecotoxicology and Environmental Safety 2009, 72 (6), 1625-1634.

(190) Caracciolo, A. B.; Grenni, P.; Sacca, M. L., Effect of the Antiviral Drug Oseltamivir (Tamiflu) on the Bacterial Community Structure of a Surface Water Ecosystem Analyzed Using Fluorescence In Situ Hybridization. Bulletin of Environmental Contamination and Toxicology 2010, 85 (5), 443-446.

(191) Slater, F. R.; Singer, A. C.; Turner, S.; Barr, J. J.; Bond, P. L., Pandemic pharmaceutical dosing effects on wastewater treatment: no adaptation of

activated sludge bacteria to degrade the antiviral drug Oseltamivir (Tamiflu (R)) and loss of nutrient removal performance. Fems Microbiology Letters 2011, 315 (1), 17-22.

(192) Fick, J.; Lindberg, R. H.; Tysklind, M.; Haemig, P. D.; Waldenstrom, J.; Wallensten, A.; Olsen, B., Antiviral Oseltamivir Is not Removed or Degraded in Normal Sewage Water Treatment: Implications for Development of Resistance by Influenza A Virus. Plos One 2007, 2 (10).

(193) Ghosh, G. C.; Nakada, N.; Yamashita, N.; Tanaka, H., Occurrence and fate of oseltamivir carboxylate (Tamiflu) and amantadine in sewage treatment plants. Chemosphere 2010, 81 (1), 13-17.

(194) Ghosh, G. C.; Nakada, N.; Yamashita, N.; Tanaka, H., Oseltamivir Carboxylate, the Active Metabolite of Oseltamivir Phosphate (Tamiflu), Detected in Sewage Discharge and River Water in Japan. Environmental Health Perspectives 2010, 118 (1), 103-107.

(195) Mestankova, H.; Schirmer, K.; Escher, B. I.; von Gunten, U.; Canonica, S., Removal of the antiviral agent oseltamivir and its biological activity by oxidative processes. Environmental Pollution 2012, 161 (0), 30-35.

(196) Accinelli, C.; Sacca, M. L.; Fick, J.; Mencarelli, M.; Lindberg, R.; Olsen, B., Dissipation and removal of oseltamivir (Tamiflu) in different aquatic environments. Chemosphere 2010, 79 (8), 891-897.

(197) Bartels, P.; von Tuempling, W., Jr., The environmental fate of the antiviral drug oseltamivir carboxylate in different waters. Science of the Total Environment 2008, 405 (1-3), 215-225.

(198) Soderstrom, H.; Jarhult, J. D.; Olsen, B.; Lindberg, R. H.; Tanaka, H.; Fick, J., Detection of the Antiviral Drug Oseltamivir in Aquatic Environments. Plos One 2009, 4 (6).

2

ANTIVIRAL DRUGS IN WASTEWATER AND SURFACE WATERS: A NEW PHARMACEUTICAL CLASS OF ENVIRONMENTAL RELEVANCE?

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Abstract

An analytical method was developed for the determination of nine antiviral drugs (acyclovir, abacavir, lamivudine, nevirapine oseltamivir, penciclovir, ribavirin, stavudine, zidovudine) and one active metabolite (oseltamivir carboxylate) in raw and treated wastewater as well as in surface water using LC/ESI tandem MS detection. Relative recoveries exceeded 80% and limits of quantification ranged between 0.2 and 10 ng L^{-1}. Matrix effects were compensated by the use of stable isotope labeled surrogate standards and optimized chromatographic separation on a Hydro-RP column. All antiviral drugs, except ribavirin, were detected in raw wastewater. A significant reduction in concentrations was observed for acyclovir, lamivudine, and abacavir in treated wastewater indicating a substantial removal, while nevirapine, zidovudine and oseltamivir were found in similar concentrations in raw and treated wastewater. Concentrations in river waters were in the lower ng L^{-1}-range, with a maximum of 190 and 170 ng L^{-1} for acyclovir and zidovudine, respectively in the Hessian Ried. Estimated total compound loads in the Ruhr river ranged between <0.1 and 16.5 g d^{-1} while for the Rhine river values between 370 and 1800 g d^{-1} were determined. The ratios of oseltamivir to oseltamivir carboxylate were approximately a factor of 10 higher for the Rhine than for the other rivers and streams indicating a significant contribution from other sources such as discharges from manufacturing facilities.

2.1 INTRODUCTION

Antiviral drugs have recently attracted the interest of the general public due to the pandemic outbreak of the swine influenza virus.[1] Besides the treatment of influenza, antivirals are administered against a broad spectrum of viral infections such as HIV, herpes, and hepatitis.[2,3] Like other pharmaceuticals, these compounds are, if not completely metabolized in patients, excreted via feces or urine. Thus, they can enter the environment via wastewater treatment plant (WWTP) discharges.[4] However, most drugs are only partly removed during wastewater treatment. For oseltamivir and its metabolite oseltamivir carboxylate it has been shown that both are not effectively removed in WWTPs.[5,6] To date, no studies have been published on the environmental occurrence and fate of other antiviral substances. However, they might be of environmental relevance as they are prescribed in amounts of up to several tons per year in Germany.[7] The environmental release of antiviral drugs is of considerable concern due to potential ecosystem alterations and the development of viral resistances.[1] Using (Q)SAR modeling of almost 3000 different compounds, Sanderson et al. suggested that antiviral drugs are among the predicted most hazardous therapeutic classes with regard to their toxicity toward algae, daphnids and fish.[8] In addition, considerable concern has been raised about the potential environmental effects of anti-influenza drugs which undergo seasonal variations, with peak emissions during influenza epidemics.[9] Their release into the environmental might cause serious effects such as the generation of oseltamivir carboxylate-resistance in wildfowl, taking into account that birds are competent hosts of influenza viruses.[10]

Based on these concerns and the potential release of large amounts of anti-influenza drugs during pandemic outbreaks, there is a need for a sensitive and reliable detection method. For the analysis of antiviral drugs in biological matrices such as blood or urine, several methods have been developed. Jung et al. used a combination of liquid-liquid extraction and protein precipitation followed by LC/MS/MS with electrospray ionization for the simultaneous analysis of 17 antiretroviral drugs in human plasma.[11] Compain et al. employed SPE for the extraction of lamivudine, stavudine and zidovudine from plasma samples.[12] Zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) SPE has been utilized by Lindegardh et al. for oseltamivir and oseltamivir carboxylate in plasma, saliva and urine.[13] SPE is also the method of choice for the analysis of antiviral drugs in environmental waters and has, for example, been employed for the detection of oseltamivir carboxylate in surface waters and wastewater.[14,15] However, the simultaneous analysis of a broad spectrum of antiviral drugs is challenging due the different chemical moieties and different pKa-values (Table 1). As a result of their high polarity, and the use of large sample volumes, necessary to achieve sufficient sensitivity, antiviral drugs are probably not effectively retained on most common SPE sorbent materials. The same is true for reversed phase

columns, generally used in liquid chromatography, which has consequences with regard to chromatographic resolution.[16] In recent years, HILIC has been successfully employed for the analysis of polar substances in biological matrices[17,18], food samples[19] and for the analysis of estrogens in river water.[20] In addition, acyclovir has been analyzed in rat plasma and tissues using HILIC.[21] In contrary to reversed-phase liquid chromatography (RPLC), polar compounds are well retained on HILIC columns, due to their interaction with the water layer formed at the surface of the stationary phase.[22]

The main objective of this study was the development and validation of a sensitive method for the simultaneous

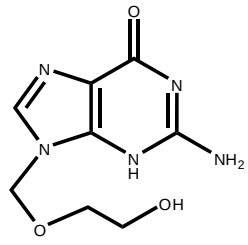
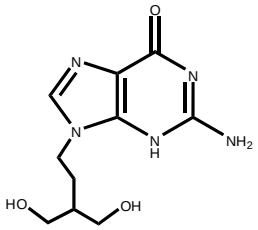
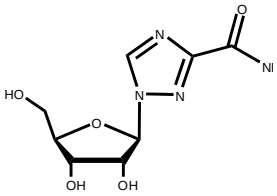
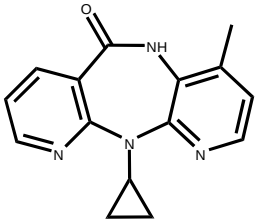
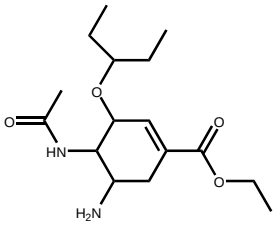
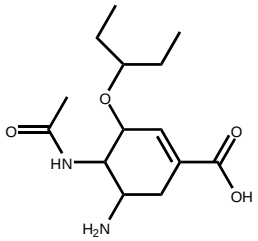
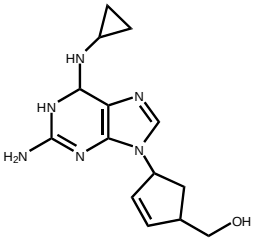
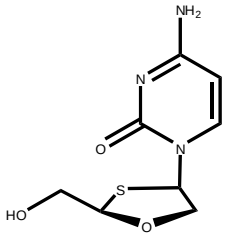
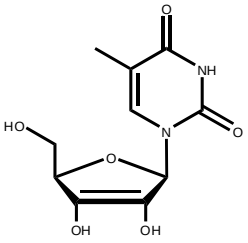
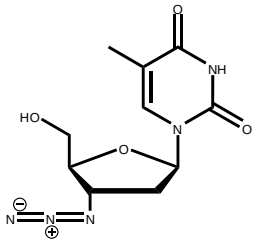
analysis of nine antiviral drugs and one active metabolite in environmental waters using SPE and LC/MS/MS. Among the nine antiviral drugs included in this study, abacavir (ABC), nevirapine (NVP), zidovudine (ZDV), lamivudine (3TC), and stavudine (d4T) are primarily used for the treatment of HIV, ribavirin (RBV) for the treatment of Hepatitis C, oseltamivir (OP) for the treatment of Influenza A and B, and acyclovir (ACV) as well as penciclovir (PCV) for the treatment of herpes infections. The method was applied to raw and treated wastewater, as well as surface waters to provide a detailed insight into the environmental occurrence of antiviral drugs in Germany at a point of no pandemic outbreak.

2.2 Materials and Methods

2.2.1 Sampling of Wastewater and Surface Water

Raw and treated wastewater were sampled from two conventional German WWTPs applying similar treatment processes, consisting of a screen, an aerated grit-removal tank and a primary clarifier followed by biological treatment (nitrification/denitrification) and chemical phosphorus removal. WWTP1 with a capacity of 330 000 population equivalents (PE) has a daily flow rate of approximately 60 000 m^3. The activated sludge system is operated with a hydraulic retention time (HRT) and sludge retention time (SRT) of approximately 12 h and 10-12 days, respectively. WWTP2 treats approximately 200 000 m^3 of wastewater per day and serves 1 350 000 PE. Biological treatment consists of two treatment units in series. HRT and SRT of the first completely aerated COD removal unit are approximately 1 h and 0.5 days, respectively. The second activated sludge unit consists of a stirred anaerobic compartment for denitrification followed by an aerated compartment for nitrification and is operated with a HRT and SRT of approximately 5 h and 18 days, respectively. Grab samples were taken from WWTP1, whereas 24 h composite samples were taken from WWTP2.

TABLE 1. Substances, CAS Numbers, Chemical Structures, Application Quantity prescribed in Germany 2007 (= number of prescribed Daily Doses x Defined Daily Doses) (*7*), respective per Capita Consumption (= application quantity prescribed/ total German population), and log K_{ow}, pK_a, pK_b

Substance, CAS-No. (Quantity prescribed)	Chemical Structure	Substance, CAS-No. (Quantity prescribed)	Chemical Structure
Acyclovir (ACV) Herpes simiplex and Varicella-zoster virus inhibitor CAS: 59277-89-3 (10.0 t; 122 µg/(cap•a)) Log K_{ow}: -1.59[25]; pK_a: 2.2 ($-NH^+$), 9.4 ($-NH_3^+$)[26]		**Penciclovir (PCV)** Herpes simiplex and Varicella-zoster virus inhibitor CAS: 39809-25-1 Log K_{ow}: -2.1[23]; pK_a: 3.2 ($-NH^+$), 9.4 ($-NH_2^+$)[24]	
Ribavirin (RBV) Hepatitis C virus inhibitor CAS: 36791-04-5 (1.1 t; 13.4 µg/(cap•a)) Log K_{ow}: -1.85[27]; pK_a: 5.9 ($-NH^+$)[28]		**Nevirapine (NVP)** Non-nucleoside reverse transcriptase inhibitor CAS: 129618-40-2 (0.7 t; 8.5 µg/(cap•a)) Log K_{ow}: 2.05[25]; pK_a: 2.8 ($-NH^+$)[29]	
Oseltamivir (OP) Neuramidase inhibitor CAS: 196618-13-0 (0.06 t; 0.7 µg/(cap•a)) Log K_{ow}: 0.36[30]; pK_a: 8.2 ($-NH_3^+$)[6]		**Oseltamivir carboxylate (OC)** metabolite of oseltamivir CAS: 187227-45-8 Log K_{ow}: -2.1[30]; pK_a: 3.6 (-COOH), 8.9 ($-NH_3^+$)[6]	
Abacavir (ABC) Nucleoside analog reverse transcriptase inhibitor CAS: 136470-78-5 (1.6 t; 19.5 µg/(cap•a)) Log K_{ow}: 0.22[25]; pK_a: 5.04 ($-NH_3^+$ or $-NH_2^+$)[25]		**Lamivudine (3TC)** Nucleoside analog reverse transcriptase inhibitor CAS: 134678-17-4 (1.7 t; 20.7 µg/(cap•a)) Log K_{ow}: 0.06[25]; pK_a: 4.4 ($-NH_3^+$)[29]	
Stavudine (d4T) Nucleoside analog reverse transcriptase inhibitor CAS: 3056-17-5 (0.06 t; 0.7 µg/(cap•a)); 2004) Log K_{ow}: -0.47[25]; pK_a: 9.8 (-NH)[29]		**Zidovudine (ZDV)** Nucleoside analog reverse transcriptase inhibitor CAS: 30516-87-1 (1.7 t; 20.7 µg/(cap•a)) Log K_{ow}: -0.1[27]; pK_a: 9.8 (-NH)[31]	

To determine the occurrence of the antivirals in rivers, two geographic regions in Germany were selected. Several rivers and streams in the Hessian Ried, as well as the Ruhr watershed, the Rhine river, and the Emscher river were monitored. The Northeastern part of the Upper Rhine Plain, known as the Hessian Ried, lies between the Mainz and Frankfurt, and has about two million inhabitants. The rivers and creeks in this area have a high proportion of treated wastewater, in some cases more than 50%. The respective sampling sites are labeled as HR_1-HR_12 (Figure 2). The river Ruhr is a medium-size river in Western Germany with a length of 217 km. The river begins in the Rothaar mountains and flows through the moderately populated

"Sauerland" area (Figure 3, km 0-117; sampling points Ru_01- Ru_17). Afterward the river flows through the Ruhr Valley (river km 117-217; sampling points Ru_18-Ru_30), Germany's most densely populated region, and joins the river Rhine in Duisburg. Wastewater of nearly two million inhabitants is discharged into the river Ruhr. In addition to the Ruhr, the river Emscher (Em_01), which receives most of the wastewater from the Ruhr Valley was monitored, as well as the Rhine before (Rh_01) and after the confluence with the Ruhr (Rh_02 and Rh_03). Grab samples were taken in amber glass bottles during a dry weather period in September 2009. All water samples were immediately transported to the lab (within 4-6 h), filtered through glass fiber filters (GF 6, <1 µm, diameter 55 mm from Schleicher and Schuell, Dassel, Germany) and acidified to pH 3 with 3.5 M sulfuric acid to inhibit microbial activity. The samples were stored at 4 °C in the dark until sample extraction (within 2-4 days).

2.2.2 Solid Phase Extraction (SPE)

For SPE 100 mL of raw wastewater, 200 mL of treated wastewater and 500 mL of surface water were spiked with 100 ng of each surrogate standard. Prior to extraction, waters samples were adjusted to pH 8 with ammonium hydroxide (25%). Isolute ENV+ cartridges (6 mL, 500 mg) were conditioned using 1 × 2 mL n-heptane, 1 × 2 mL acetone, 3 × 2 mL methanol followed by 4 × 2 mL groundwater (pH 8). Samples were passed through the preconditioned cartridges at a flow rate of approximately 5 mL min^{-1}. Subsequently, cartridges were dried under a gentle stream of nitrogen. The analytes were eluted with 5×2mL methanol/acetone 50/50+0.2% formic acid. Sample extracts were evaporated to 100 µL under a gentle stream of nitrogen and reconstituted to 1mL with the aqueous buffer (5 mM NH_4-formate).

2.2.3 LC-MS Analysis

The HPLC system consisted of a G1367C autosampler, a G1312B binary HPLC pump, a G1379B degasser (all Agilent 1200 SL Series, Waldbronn, Germany) and a MistraSwitch column oven (MayLab Analytical Instruments, Vienna, Austria). For chromatographic separation two different approaches (RPLC and HILIC) were initially tested. However, the following only discusses the RPLC approach. Detailed information about the HILIC method is found in the Supporting Information (SI).

For the RPLC method, a 4 µm Synergi Hydro-RP (150 × 3 mm) column protected by a SecurityGuard column (4 × 3mm i.d.; Phenomenex, Aschaffenburg, Germany) was used. An aliquot of 25 µL of each sample was injected into the LC/MS/MS system using 5 mM NH_4-formate (A) and methanol (B) as mobile phases. A gradient program was used to achieve separation of the analytes, the percentage of (A) was changed linearly as follows: 0-4 min, 100%; 7 min, 30%; 17 min, 10%; 18 min, 100%. The run time was 22 min, flow rate was 0.4 mL min^{-1}, and column oven temperature was set to 40 °C.

The HPLC system was coupled to an API-4000 Q Trap (Applied Biosystems/MDS Sciex, Darmstadt, Germany). Analytes were quantified in multiple reaction monitoring mode (MRM) using electrospray ionization-mass spectrometry (ESI-MS) with Turbo Ionspray in positive mode. The two most intense MRM transitions for each substance were monitored for identification and quantification of the analytes. MS/MS parameters were optimized in continuous flow mode, injecting 500 ng mL^{-1} standard solutions dissolved in methanol at a flow rate of 10 µL min^{-1}. Declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were optimized in the autotuning program of the Analyst software (Version 1.4.2.). Retention times of the analytes as well as optimized MS parameters are listed in SI Table S1.

MRM transitions were measured in time periods since (i) higher sensitivity is obtained due to increased MRM dwell times and (ii) allows for the individual optimization of ESI source parameters for each period. In period 1 (0-9.0 min), ribavirin was measured with a dwell time of 300 ms. In period 2 (9.0-10.4 min), acyclovir, lamivudine, penciclovir, and stavudine were measured with a dwell time of 80 ms. In period 3 (10.4-22.0 min), abacavir, nevirapine, oseltamivir, oseltamivir carboxylate, and zidovudine were measured with a dwell time of 100 ms. For period 2 the following ESI source parameters were used: CUR, 15 psi; GS1, 20 psi; GS2, 10 psi. For period 1 and 3 the following parameters were used: CUR, 35 psi; GS1, 45 psi; GS2, 45 psi. All other source parameters showed no or only negligible effects on signal intensities and were therefore kept constant (collision gas, high; ion spray voltage, 5,500 V; source temperature, 500 °C; entrance potential, 10 V).

2.2.4 QUANTIFICATION

Calibration curves (11 points) were prepared by spiking 500mL of pristine groundwater with the analytes at concentrations of 0.2, 1, 2, 4, 10, 20, 50, 100, 200, 400, and 1000 ng L^{-1}. A constant amount of surrogate standards (100 ng) was added. Samples were then subjected to SPE as described above. Ribavirin-^{13}C5, acyclovir-d4, penciclovir-d4, ^{13}C-^{15}N2-lamivudine, stavudine-d3, zidovudine-d3, and nevirapine-d5 were used as surrogate standards. Quantification of abacavir was performed using nevirapine-d5, while for oseltamivir and oseltamivir carboxylate stavudine-d3 and ^{13}C,^{15}N2-lamivudine were used, respectively. Linear regression was applied to the calibration curves with a weighing factor 1/x.

2.2.5 METHOD VALIDATION

The sensitivity of the method was evaluated by determining the limit of quantification (LOQ) for all analytes in the calibration standards using 500 mL of groundwater spiked with different amounts of analytes prior to extraction. The LOQ was defined as the lowest calibration point in the linear regression with a signal-to-noise (S/N) of 10 for the most sensitive transition (MRM1) and a S/N of 3 for the second transition(MRM2).

The accuracy of the method was determined by spiking known amounts of analytes into surface water, WWTP influent and effluent samples (five replicates each). Relative recoveries were calculated by dividing the measured quantities with the nominal (spiked) quantities. Depending on the background concentrations of the analytes in the respective matrix, different quantities (5, 50, or 200 ng) were spiked into the water samples. However, for each matrix at least two different amounts were added. Blanks subjected to the entire preparation and analysis procedure were performed. Deviations from mean values are given as 95% confidence intervals.

One of the major drawbacks of ESI-MS are matrix effects caused by coeluting substances resulting in either signal suppression or enhancement. Matrix effects of electrospray ionization were determined in the different matrices (surface water, WWTP influent, WWTP effluent). The matrix effect (ME) was calculated as the ratio of the analyte peak area in the sample spiked after SPE ($PA_{postspike}$) subtracted by the peak area in the nonspiked blank sample (PA_{blank}) to the peak area in a nonenriched external standard (PA_{st}):

$$ME[\%] = \frac{\left(PA_{post-spike} - PA_{blank}\right)}{PA_{st}} \cdot 100$$

A value of 100% indicates that there are no matrix effects, whereas values <100% or >100% indicate signal suppression or enhancement, respectively. All values were corrected for blank concentrations.

2.3 RESULTS AND DISCUSSION

2.3.1 ANALYTICAL METHOD

The 500 mg Isolute ENV+ at pH 8 with an elution solvent of methanol/acetone 50/50+0.2% formic acid showed the best results, with absolute recoveries ranging from 66 to 119% (SI Table S2). Due to the high polarity of the antiviral drugs, the 500 mg ENV+ sorbent led to significantly higher recoveries than 200 mg sorbent. Concerning RPLC, the variation of the mobile phase composition, used for chromatographic separation of the analytes, revealed that the addition of ammonium formate, ammonium acetate, and formic acid exhibited negative effects on the ionization of the analytes. However, without buffer addition, peak broadening and instability of retention times were observed for several compounds. As a compromise 5 mM NH_4 formate aqueous buffer (pH 5.6) was used. Calibration curves showed excellent linearity over the whole calibration range with correlation coefficients >0.99 for all analytes (Table 2). Relative recoveries varied between 88 ± 13 and 108 ± 3% for surface water, between 92 ± 7 and 135 ± 20% for WWTP effluent samples and between 83 ± 3 and 105 ± 9% for WWTP influent samples. The limits of quantification (LOQ) of the analytical method ranged between 0.2 and 10 ng L^{-1}. Respective LOQs for WWTP influents and effluents were assessed by calculation of the S/N ratios of both MRM transitions using either the background concentrations of the analytes or a total spiked amount of 5 ng (SI Table S3). Considering the difference of sample volumes enriched (500 mL for river water, 200 mL for WWTP influent, and 100 mL for WWTP effluent) the LOQs for WWTP influent and effluent were multiplied by a factor of 5 and 2.5, respectively, in comparison to the LOQs attained for groundwater. Only for lamivudine the factor for WWTP influent and effluent samples was 10 and 5, respectively, due to a low S/N ratio of the second MRM transition.

2.3.2 MATRIX EFFECTS

All investigated substances are prone to matrix effects but to various extents (SI Figure S4). In general, matrix effects increase in the following order: surface water < WWTP effluent < WWTP influent for both RPLC and HILIC. For most of the analytes higher matrix effects were observed for HILIC with a clear correlation of retention times and matrix effects being present. For this reason it was decided to use RPLC for chromatographic separation. To compensate for matrix effects, different approaches can be employed such as the use of labeled analogues of the analytes as surrogate standards, alternative ionization techniques or additional sample cleanup methods. In the described analytical method, seven ^{13}C-, ^{15}N-, or D-labeled surrogate standards were used. All other approaches to reduce matrix effects were not successful. The application of atmospheric pressure chemical ionization (APCI), which has been shown to be less susceptible to matrix effects was inappropriate.[32] Compared to ESI, signal

intensities of the selected substances were at least 50% lower and several analytes were not or only marginally ionized (SI Figure S1). Since APCI is mostly applicable to substances with a lower polarity, the low ionization of the highly polar antiviral drugs can be expected.[33] Additional cleanup steps such as size exclusion chromatography (SEC) exhibited no significant reduction in matrix effects (Phenogel 50 Å, Phenomenex, Aschaffenburg, Germany; THF and acetone were tested). Furthermore, SEC caused high losses of the analytes. A derivatization using dansyl chloride, which has been employed, for example, for structurally related amides, was inappropriate for the antiviral drugs selected.[34] Dansyl chloride was obviously not reacting with amide nitrogens and nitrogens which were included in the conjugated moiety.

TABLE 2. Linearity of calibration curves, limit of quantification and recoveries in the investigated matrices with 95% confidence intervals after subtracting the background concentration. For calculation of relative recoveries, different amounts (5, 50 or 200 ng) of analytes were added depending on the sampled matrix.

Analyte	Linearity (r^2)	Rhine water			WWTP effluent			WWTP influent		
		LOQ [ng L^{-1}]	absolute recovery[a] [%]	relative recovery[b] [%]	LOQ [ng L^{-1}]	absolute recovery[a] [%]	relative recovery[b] [%]	LOQ [ng L^{-1}]	absolute recovery[a] [%]	relative recovery[b] [%]
Abacavir	0.9940	0.2	55 ± 2	108 ± 3	0.5	41 ± 1	100 ± 3	1	28 ± 1	83 ± 3
Acyclovir	0.9994	1	85 ± 9	104 ± 4	2.5	66 ± 6	101 ± 7	5	62 ± 9	95 ± 13
Lamivudine	0.9994	10	73 ± 17	100 ± 10	50	47 ± 1	95 ± 14	100	37 ± 1	99 ± 9
Nevirapine	0.9992	1	65 ± 2	106 ± 4	2.5	42 ± 1	106 ± 3	5	30 ± 1	99 ± 5
Oseltamivir	0.9989	0.2	70 ± 6	101 ± 6	0.5	70 ± 6	135 ± 20	1	35 ± 4	96 ± 16
Oseltamivir carboxylate	0.9987	0.2	37 ± 6	88 ± 13	0.5	47 ± 8	111 ± 13	1	32 ± 1	105 ± 9
Penciclovir	0.9988	1	82 ± 4	101 ± 6	2.5	61 ± 9	104 ± 8	5	61 ± 4	98 ± 13
Ribavirin	0.9996	4	16 ± 2	101 ± 4	10	32 ± 1	100 ± 9	20	53 ± 6	104 ± 5
Stavudine	0.9990	1	92 ± 6	95 ± 5	2.5	39 ± 22	95 ± 6	5	42 ± 8	87 ± 17
Zidovudine	0.9999	1	80 ± 10	100 ± 3	2.5	54 ± 3	92 ± 7	5	29 ± 3	95 ± 6

[a] Calculated in comparison to a non-enriched standard solution (n = 3); [b] Calculated using the recovery of the spiked surrogate standard (n = 10)

2.3.3 Occurrence of Antiviral Drugs in Environmental Samples

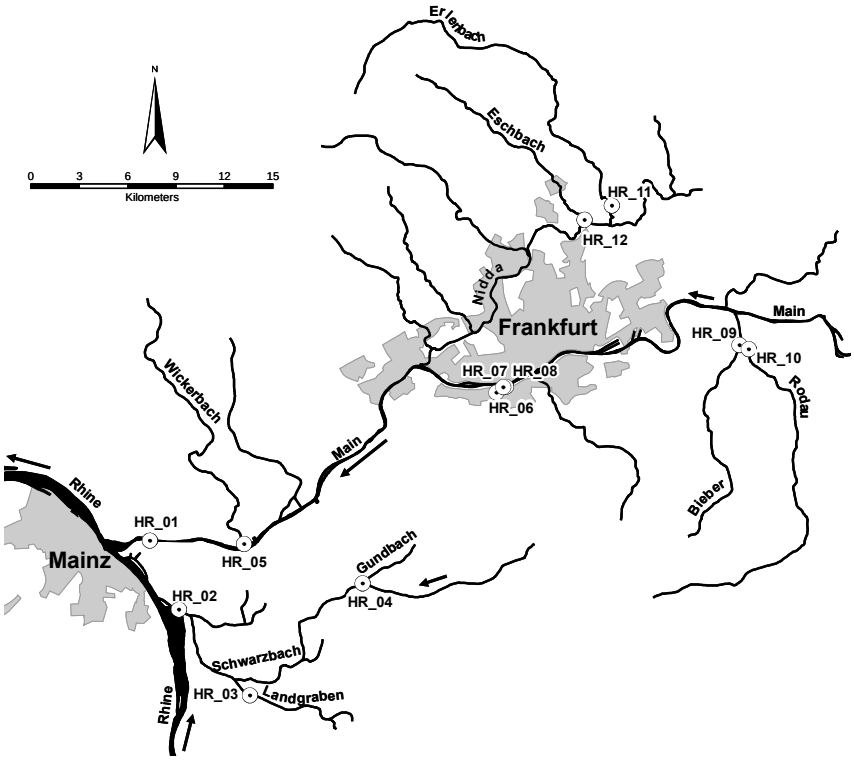
WWTPs. All antiviral drugs, with the exception of ribavirin, were detected in influents of at least one of the two WWTPs sampled (Table 3). Highest concentrations were observed for acyclovir, lamivudine, zidovudine and abacavir with concentrations up to 1800, 720, 380, and 220 ng L^{-1}, respectively. Concentrations of all other analytes were lower than 50 ng L^{-1}. Results from the 24 h composite samples collected from WWTP2 indicate an almost complete removal of abacavir, acyclovir, lamivudine, penciclovir, and stavudine ranging from 87 to >99%. With the exception of zidovudine, similar removal rates were calculated for WWTP1, although only grab samples were taken. Elevated concentrations of zidovudine in the effluents of WWTP2 might be caused by the cleavage of glucuronide conjugates known to be excreted by humans up to 70%.[35] There are no reasonable explanations for the elevated nevirapine concentrations in the WWTP effluent. However, it cannot be excluded that the confidence interval calculated underestimates the real statistical uncertainties of the nevirapine concentrations, and thus the increase is artificial.

TABLE 3. Antiviral drugs in influents and effluents of sampled WWTPs. Statistical uncertainties: 95% confidence intervals (n=3).

	WWTP1 (grab samples)			WWTP2 (24 h composite samples)		
	concentration [ng L^{-1}]		elimination [%]	concentration [ng L^{-1}]		elimination [%]
Analyte	**Influent**	**Effluent**		**Influent**	**Effluent**	
Ribavirin	<LOQ	<LOQ	-	<LOQ	<LOQ	-
Acyclovir	1780 ± 99	27.3 ± 1.6	98	1780 ± 50	53.3 ± 3.5	97
Penciclovir	42.8 ± 9.8	<LOQ	>94	19.5 ± 9.7	<LOQ	>87
Lamivudine	210 ± 13	<LOQ	>76	720 ± 130	<LOQ	>93
Stavudine	11.6 ± 5.3	<LOQ	>78	22.8 ± 5.6	<LOQ	>89
Abacavir	81.7 ± 5.3	<LOQ	>99	225 ± 40	<LOQ	>99
Nevirapine	4.8 ± 2.9	7.2 ± 2.3	none	21.8 ± 2.3	32.1 ± 0.5	none
Oseltamivir	11.9 ± 4.2	15.8 ± 4.0	none	< LOQ	8.9 ± 0.7	none
Oseltamivir carboxylate	42.7 ± 9.3	17.3 ± 5.4	59	29.4 ± 4.9	12.2 ± 2.9	59
Zidovudine	310 ± 22	98.2 ± 17.6	68	380 ± 14	564 ± 22	none

Hessian Ried. In rivers and streams from the Hessian Ried, maximum concentrations were found for acyclovir and zidovudine with concentrations up to 190 ng L^{-1} (Erlenbach; HR_11) and 170 ng L^{-1} (Bieber; HR_09), respectively (Figure 1). Ribavirin and lamivudine were not detected at all, penciclovir was determined with concentrations close to the LOQ in the Landgraben (HR_03), and abacavir was present only in the Erlenbach (HR_11). These results are in good agreement with results from the two WWTPs with penciclovir, lamivudine, stavudine and abacavir being present in WWTP influents but not detected in WWTP effluents. Probably due to the limited biological transformation (e.g., during wastewater treatment and in rivers) oseltamivir and its active metabolite, oseltamivir carboxylate, were detected in almost all of the surface water samples collected in the Hessian Ried with maximum concentrations of 15 and 24 ng L^{-1}, respectively.

Ruhr Watershed. For most of the antiviral drugs analyzed, concentrations were higher at sampling sites further downstream along the Ruhr (i.e., increasing in distance from the headwaters) (Figure 2). Highest concentrations were found downstream of WWTP discharges with concentrations up to 29 and 20 ng L^{-1} for zidovudine and acyclovir, respectively. Compared to the Rhine, concentrations of antiviral drugs were higher in the Ruhr with the exception of oseltamivir for which concentrations of up to 17 ng L^{-1} were determined in the Rhine, compared to a maximum concentration of 2.2 ng L^{-1} in the Ruhr. Even in the Emscher, which receives WWTP discharges from around 4.5 million people, concentrations of oseltamivir did not exceed 2.9 ng L^{-1}. However, the concentrations of the metabolite oseltamivir carboxylate were comparable to those observed in the Ruhr, Rhine and Emscher.

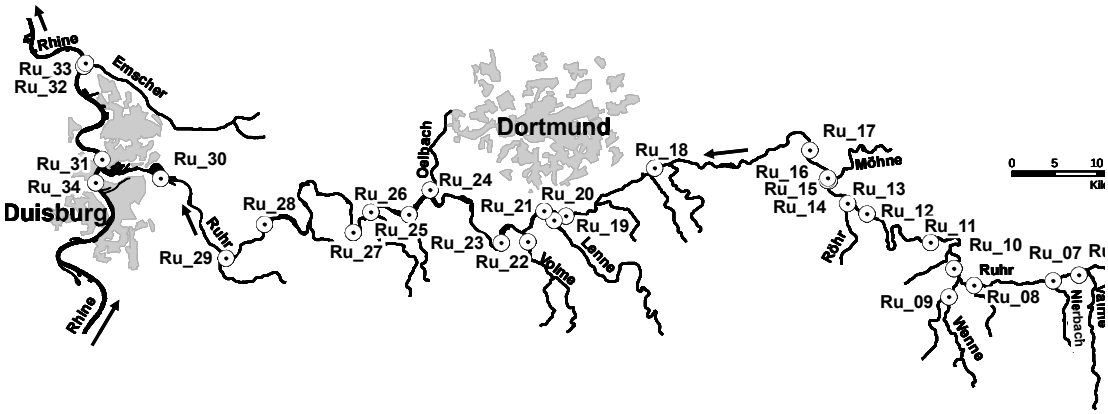


sampling location	ACV	PCV	d4T	ABC	NVP	OP	OC	ZDV
HR_01	7.0 ± 0.9	< LOQ	< LOQ	< LOQ	< LOQ	1.9 ± 0.9	< LOQ	18 ± 2
HR_02	5.6 ± 1.9	< LOQ	2.9 ± 0.2	< LOQ	10 ± 1	6.6 ± 0.1	2.1 ± 0.5	67 ± 1
HR_03	4.4 ± 0.8	7.0 ± 1.8	< LOQ	< LOQ	13 ± 1	6.4 ± 2.2	3.1 ± 0.8	61 ± 3
HR_04	2.2 ± 0.2	< LOQ	< LOQ	< LOQ	6.3 ± 0.3	2.1 ± 0.8	1.9 ± 0.5	60 ± 8
HR_05	86 ± 5	< LOQ	< LOQ	< LOQ	5.4 ± 0.3	15 ± 2	21 ± 1	62 ± 3
HR_07	3.7 ± 0.5	< LOQ	2.0 ± 0.7	< LOQ	< LOQ	0.6 ± 0.5	< LOQ	4.5 ± 0.5
HR_08	4.0 ± 0.5	< LOQ	2.3 ± 0.2	< LOQ	< LOQ	1.0 ± 0.7	0.6 ± 0.4	4.5 ± 0.3
HR_09	22 ± 2	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	170 ± 3
HR_10	15 ± 1	< LOQ	< LOQ	< LOQ	17 ± 1	15 ± 6	4.8 ± 2.0	96 ± 3
HR_11	190 ± 15	< LOQ	< LOQ	1.4 ± 0.4	< LOQ	14 ± 5	24 ± 2	60 ± 2
HR_12	44 ± 3	< LOQ	< LOQ	< LOQ	< LOQ	6.3 ± 0.8	5.0 ± 0.4	22 ± 1

FIGURE 1. Occurrence of antiviral drugs in rivers and streams in the Hessian Ried Region, south of Frankfurt am Main, Germany (LOQ: limit of quantification). Mean concentrations [ng L^{-1}] are given together with 95% confidence intervals (n = 3).

The increase of antiviral loads after river location Ru_18 can be attributed to the discharge of WWTP effluents. Similar results in the Ruhr were observed by Andresen et al. for organophosphorous flame retardants and plasticizers.[36] The decrease of acyclovir loads within the urban area (from Ru_18 to Ru_30) is an indication for its removal in the river. Loads of the other antiviral drugs (zidovudine, oseltamivir, oseltamivir carboxylate) remained fairly stable. As the measured and predicted loads were also comparable (SI Table S8), zidovudine, oseltamivir, and oseltamivir carboxylate are obviously not degraded or sorbed to appreciable proportions in the Ruhr. In addition, the ratio of oseltamivir and oseltamivir carboxylate (OP/OC ratio, see SI Table S7) was calculated. The OP/OC ratio suggests if other sources than municipal WWTP effluents are responsible for the surface water contamination (e.g., via a direct discharge of OP by a manufacturer) or if both are degraded to a different extent. In raw wastewater a mean OP/OC ratio of 0.3 was determined, which can be explained by the metabolism of oseltamivir in the human body yielding 70-80% oseltamivir carboxylate. In treated wastewater the ratios were 0.72 ± 0.23 for WWTP2 and 0.91 ± 0.20 for WWTP1, due to the reduced concentrations of oseltamivir carboxylate and the stability of oseltamivir (see Table 3). In the Ruhr and the Hessian area the OP/OC ratios ranged from 0.34-1.48 and 0.57-3.10, respectively. Based on the results discussed above, it is indicated that these elevated OP/OC ratios are primarily caused by the degradation of oseltamivir carboxylate in WWTPs, which is in contrast to previous studies (5, 6), whereas its degradation in surface waters is obviously only of minor importance.[37-39]

In the river Rhine significantly higher OP/OC ratios (13.8 ± 3.5) were found due to elevated oseltamivir concentrations of up to 17 ng L^{-1} indicating the contribution of another source besides WWTPs discharges. To elucidate the origin of oseltamivir in the Rhine additional samples were taken at Weil, which is located in Southern Germany close to Switzerland and France. This international monitoring station offers the possibility to sample five different sites across the river profile (Figure 3). Oseltamivir concentrations increased sharply from the Western (sampling point S5) to the Eastern location (sampling point S1), with concentrations up to 160 ng L^{-1} and a mean OP/OC-ratio of 13.1 (SI Table S7). On the other hand a homogeneous distribution was observed in the five monitoring sites for the other antiviral drugs. Approximately two km upstream, oseltamivir and oseltamivir carboxylate were not detected above the LOQ (results not shown).



river location	Mean daily discharge at sampling date [$m^3 s^{-1}$]	Acyclovir		Zidovudine		Oseltamivir		Oseltamivir carboxylate	
		Conc. [ng L^{-1}]	load [g d^{-1}]	Conc. [ng L^{-1}]	load [g d^{-1}]	Conc. [ng L^{-1}]	load [g d^{-1}]	Conc. [ng L^{-1}]	load [g d^{-1}]
				Ruhr					
Ru_07	5.5	2.6	0.8	2.1	0.7	0.8	0.3	1.3	0.4
Ru_11	5.9	4.1	1.7	1.2	0.5	0.3	0.1	0.6	0.3
Ru_18	13.7	7.1	8.3	2.3	2.7	0.4	0.4	<LOQ	-
Ru_23	21.5	8.9	17	7.6	14	0.6	1.1	<LOQ	-
Ru_27	23.2	6.4	13	6.2	13	1.0	2.0	0.9	1.8
Ru_30	23.2	7.0	14	7.3	15	0.9	1.8	0.6	1.2
				Ruhr tributaries					
Wenne	1.2	3.5	0.4	3.1	0.3	0.3	0.03	0.8	0.08
Lenne	11.3	20	20	6.2	6.2	0.6	0.6	1.5	1.5
Volme	1.3	6.1	0.7	4.8	0.5	1.0	0.1	0.8	0.09
Emscher	8.5	31	22	94	69	2.9	2.1	2.4	1.8
Rhine	1240	3.6	390	5.1	540	17	1800	1.3	142

FIGURE 2. Concentrations and estimated loads of antiviral drugs in the Ruhr river, its major tributaries, as well as the Emscher and the Rhine. For calculations of loads only those locations were used for which reliable discharge data were available. Further details about sampling locations and detected concentrations are provided in Supporting Information Tables S5 and Table S6.

The total load of oseltamivir at Weil was 2.4 kg d^{-1} which is in good agreement with the load calculated at the inflow of the Ruhr into the Rhine 610 km downstream (1.8 kg d^{-1}). These results confirm that discharges of a manufacturer located in the vicinity are probably responsible for these elevated oseltamivir concentrations. Currently the ecotoxicological risks resulting from the occurrence of antiviral drugs in the environment cannot be assessed due to the lack of published effect data on aquatic organisms.

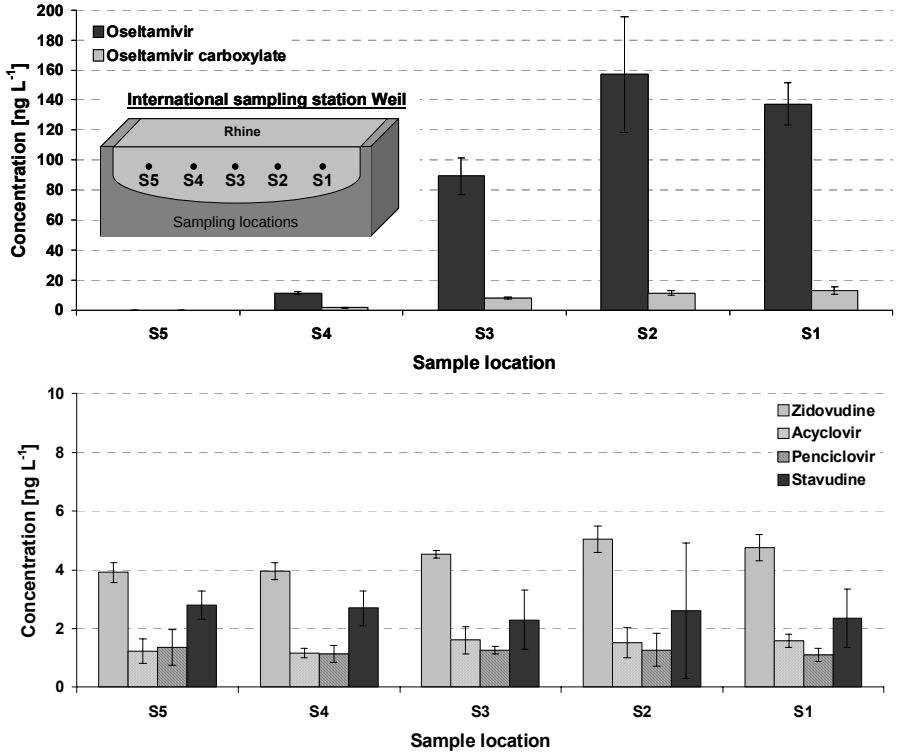


FIGURE 3. Concentrations of antiviral drugs across the river transect at the International Rhine Water Monitoring Station at Weil (west to east from S5 to S1)

2.4 REFERENCES

(1) Singer, A. C.; Nunn, M. A.; Gould, E. A.; Johnson, A. C. Potential risks associated with the proposed widespread use of Tamiflu. Environ. Health Perspect. 2007, 115 (1), 102–106.

(2) De Clercq, E. Strategies in the design of antiviral drugs. Nat. Rev. Drug Discov. 2002, 1 (1), 13–25.

(3) De Clercq, E. Three decades of antiviral drugs. Nat. Rev. Drug Discov. 2007, 6 (12), 941–941.

(4) Kümmerer, K., Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks., 3 ed.; Springer: Berlin, Heidelberg, 2008.

(5) Fick, J.; Lindberg, R. H.; Tysklind, M.; Haemig, P. D.; Waldenström, J.; Wallensten, A.; Olsen, B. Antiviral oseltamivir is not removed or degraded in normal sewage water treatment: implications for development of resistance by influenza A virus. PLoS ONE 2007, 2 (10), e986.

(6) Straub, J. O. An environmental risk assessment for oseltamivir (Tamiflu®) for sewage works and surface waters under seasonal influenza- and pandemic-use conditions. Ecotoxicol. Environ. Saf. 2009, 72 (6), 1625–1634.

(7) Schwabe, U.; Paffrath, D., Arzneiverordnungs-Report 2008; Springer: Berlin, Heidelberg, 2008.

(8) Sanderson, H.; Johnson, D. J.; Reitsma, T.; Brain, R. A.; Wilson, C. J.; Solomon, K. R. Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. Regul. Toxicol. Pharmacol. 2004, 39 (2), 158–183.

(9) Singer, A. C.; Howard, B. M.; Johnson, A. C.; Knowles, C. J.; Jackman, S.; Accinelli, C.; Caracciolo, A. B.; Bernard, I.; Bird, S.; Boucard, T.; et al. Meeting report: Risk assessment of Tamiflu use under pandemic conditions. Environ. Health Perspect. 2008, 116 (11), 1563–1567.

(10) Olsen, B.; Munster, V. J.; Wallensten, A.; Waldenstrom, J.; Osterhaus, A.; Fouchier, R. A. M. Global patterns of influenza A virus in wild birds. Science 2006, 312 (5772), 384–388.

(11) Jung, B. H.; Rezk, N. L.; Bridges, A. S.; Corbett, A. H.; Kashuba, A. D. M. Simultaneous determination of 17 antiretroviral drugs in human plasma for quantitative analysis with liquid chromatography-tandem mass spectrometry. Biomed. Chromatogr. 2007, 21 (10), 1095–1104.

(12) Compain, S.; Schlemmer, D.; Levi, M.; Pruvost, A.; Goujard, C.; Grassi, J.; Benech, H. Development and validation of a liquid chromatographic/tandem mass spectrometric assay for the quantitation of nucleoside HIV reverse transcriptase inhibitors in biological matrices. J. Mass Spectrom. 2005, 40 (1), 9–18.

(13) Lindegardh, N.; Hanpithakpong, W.; Wattanagoon, Y.; Singhasivanon, P.; White, N. J.; Day, N. P. J. Development and validation of a liquid chromatographic-tandem mass spectrometric method for determination of oseltamivir and its metabolite oseltamivir carboxylate in plasma, saliva and urine. J. Chromatogr., B 2007, 859 (1), 74–83.

(14) Söderström, H.; Ja¨rhult, J. D.; Olsen, B.; Lindberg, R. H.; Tanaka, H.; Fick, J. Detection of the antiviral drug oseltamivir in aquatic environments. PLoS ONE 2009, 4 (6), e6064.

(15) Ghosh, G. C.; Nakada, N.; Yamasjita, N.; Tanaka, H., Oseltamivir carboxylate - the active metabolite of oseltamivir phosphate (Tamiflu), detected in sewage discharge and river water in Japan. Environ. Health Perspect. [Online early access] DOI: http:// dx.doi.org/10.1289/ehp.0900930. Published online: September 28, 2009 (accessed October 7, 2009).

(16) Hemstrom, P.; Irgum, K. Hydrophilic interaction chromatography. J. Sep. Sci. 2006, 29 (12), 1784–1821.

(17) Macek, J.; Ptacek, P.; Klima, J. Determination of tolterodine and its 5-hydroxymethyl metabolite inhumanplasma by hydrophilic interaction liquid chromatography-tandem mass spectrometry. J. Chromatogr., B 2009, 877 (10), 968–974.

(18) Oertel, R.; Arenz, N.; Pietsch, J.; Kirch, W. Simultaneous determination of three anticonvulsants using hydrophilic interaction LC-MS. J. Sep. Sci. 2009, 32 (2), 238–243.

(19) Esparza, X.; Moyano, E.; Galceran, M. T. Analysis of chlormequat and mepiquat by hydrophilic interaction chromatography coupled to tandem mass spectrometry in food samples. J. Chromatogr., A 2009, 1216 (20), 4402–4406.

(20) Qin, F.; Zhao, Y. Y.; Sawyer, M. B.; Li, X. F. Column-switching reversed phase-hydrophilic interaction liquid chromatography/ tandem mass spectrometry method for determination of free estrogens and their conjugates in river water. Anal. Chim. Acta 2008, 627 (1), 91–98.

(21) Brown, S. D.; White, C. A.; Bartlett, M. G. Hydrophilic interaction liquid chromatography/electrospray mass spectrometry determination of acyclovir in pregnant rat plasma and tissues. Rapid Commun. Mass Spectrom. 2002, 16 (19), 1871–1876.

(22) Ikegami, T.; Tomomatsu, K.; Takubo, H.; Horie, K.; Tanaka, N. Separation efficiencies in hydrophilic interaction chromatography. J. Chromatogr., A 2008, 1184 (1-2), 474–503.

(23) Kezic, S.; Nielsen, J. B. Absorption of chemicals through compromised skin. Int. Arch. Occup. Environ. Health 2009, 82 (6), 677–688.

(24) Virtual Computational Chemistry Laboratory (VCCLAB)ALOGPS Database http://www.vcclab.org/ lab/alogps/start.html (accessed May 20, 2009).

(25) Kasim, N. A.; Whitehouse, M.; Ramachandran, C.; Bermejo, M.; Lennernas, H.; Hussain, A. S.; Junginger, H. E.; Stavchansky, S. A.; Midha, K. K.; Shah, V. P.; et al. Molecular properties of WHO essential drugs and provisional biopharmaceutical classification. Mol. Pharmacol. 2004, 1 (1), 85–96.

(26) Laskin, O. L. Clinical Pharmacokinetics of Acyclovir. Clin. Pharmacokinet. 1983, 8 (3), 187–201.

(27) Syracuse SRC Interactive PhysProp Database. http://www.syrres.com/ esc/physdemo.htm (accessed May 20, 2009).

(28) Wu, J. Z.; Walker, H.; Lau, J. Y. N.; Hong, Z. Activation and deactivation of a broad-spectrum antiviral drug by a single enzyme: Adenosine deaminase catalyzes two consecutive deamination reactions. Antimicrob. Agents Chemother. 2003, 47 (1), 426–431.

(29) Sekar, R.; Azhaguvel, S. MEKC determination of antiretroviral reverse transcriptase inhibitors lamivudine, stavudine, and nevirapine in pharmaceutical formulations. Chromatographia 2008, 67 (5-6), 389–398.

(30) Oo, C.; Hill, G.; Dorr, A.; Liu, B.; Boellner, S.; Ward, P. Pharmacokinetics of anti-influenza prodrug oseltamivir in children aged 1-5 years. Eur. J. Clin. Pharmacol. 2003, 59 (5- 6), 411–415.

(31) Checa, A.; Soto, V. G.; Hernandez-Cassou, S.; Saurina, J. Fast determination of pK(a) values of reverse transcriptase inhibitor drugs for AIDS treatment by using pH-gradient flow-injection analysis and multivariate curve resolution. Anal. Chim. Acta 2005, 554 (1-2), 177–183.

(32) Lien, G. W.; Chen, C. Y.; Wang, G. S. Comparison of electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization for determining estrogenic chemicals in water by liquid chromatography tandem mass spectrometry with chemical derivatizations. J. Chromatogr., A 2009, 1216 (6), 956–966.

(33) Kloepfer, A.; Quintana, J. B.; Reemtsma, T. Operational options to reduce matrix effects in liquid chromatography-electrospray ionization-mass spectrometry analysis of aqueous environmental samples. J. Chromatogr., A 2005, 1067 (1-2), 153–160.

(34) Guo, K.; Li, L. Differential C-12/C-13-isotope dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome. Anal. Chem. 2009, 81 (10), 3919–3932.

(35) Veal, G. J.; Back, D. J. Metabolism of zidovudine. Gen. Pharmacol. 1995, 26 (7), 1469–1475.

(36) Andresen, J. A.; Grundmann, A.; Bester, K. Organophosphorus flame retardants and plasticisers in surface waters. Sci. Total Environ. 2004, 332 (1-3), 155–166.

(37) Accinelli, C.; Caracciolo, A. B.; Grenni, P. Degradation of the antiviral drug oseltamivir carboxylate in surface water samples. Int. J. Environ. Anal. Chem. 2007, 87 (8), 579–587.

(38) Bartels, P.; von Tumpling, W. The environmental fate of the antiviral drug oseltamivir carboxylate in different waters. Sci. Total Environ. 2008, 405 (1-3), 215–225.

(39) Sacca, M. L.; Accinelli, C.; Fick, J.; Lindberg, R.; Olsen, B. Environmental fate of the antiviral drug Tamiflu in two aquatic ecosystems. Chemosphere 2009, 75 (1), 28–33.

3

OCCURRENCE OF OSELTAMIVIR (TAMIFLU®), OSELTAMIVIR CARBOXYLATE AND OTHER ANTIVIRAL DRUGS IN THE RIVER RHINE DURING THE SWINE FLU PANDEMIC IN NOVEMBER/ DECEMBER 2009

Parts have been published in

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Carsten Prasse, Ralf Schulz and Thomas A. Ternes

(short communication; in German language).

Abstract

A monitoring campaign of antiviral drugs in the river Rhine during the 2009 worldwide swine flu pandemic was conducted by taking 24h composite samples over a period of 43 days. For oseltamivir and oseltamivir carboxylate concentrations up to 48 and 20 ng L^{-1}, respectively, were observed corresponding to river loads of 4.6 and 2.2 kg d^{-1}, respectively. Furthermore, calculated oseltamivir to oseltamivir carboxylate ratios (OP/OC ratios) indicated the intermittent influence of two principal sources for both compounds with industrial discharges predominating at the beginning of the sampling campaign whereas excretions of individuals treated with oseltamivir were primarily responsible for observed river loads of both compounds during the pandemic. Furthermore, a good agreement between river loads of oseltamivir carboxylate and the reported number of swine flu cases was observed, indicating that oseltamivir carboxylate can be used as an indicator for a swine flu epidemic in surface waters. Analysis of other antiviral drugs revealed also the constant presence of acyclovir and zidovudine with average concentrations of 3.7 and 5.9 ng L^{-1}, respectively. Elevated river loads of both compounds during periods with higher river discharges indicate that discharge of untreated wastewater during rain events.

3.1 INTRODUCTION

Antiviral drugs such as acyclovir (Zovirax®) and oseltamivir (Tamiflu®) have recently been detected in surface waters at concentrations in the low ng/L-range.[1] Especially for oseltamivir and its active metabolite oseltamivir carboxylate, used for the treatment of (swine) influenza, considerable concern has been raised regarding the potential development of viral resistances if these compounds are emitted into the aquatic environment.[2, 3] Several studies so far have shown that both oseltamivir and oseltamivir carboxylate are only insufficiently eliminated during wastewater treatment.[1, 4, 5] Thus, during flu epidemics high loads of both compounds can be expected to be emitted into surface waters. After the release into the environment both compounds are only marginally eliminated resulting in a high environmental stability of both compounds.[6, 7] Only in shallow and clear surface waters also photodegradation can contribute to the degradation of both compounds even though only to a limited extent.[8, 9] As a result, concentrations of oseltamivir carboxylate in surface waters during the 2008/2009 flu season epidemic in Japan up to 190 ng L^{-1} were observed.[10, 11]

As up to now a comprehensive study on the occurrence of anti-influenza drugs during a pandemic is lacking, the aim of this study was i) to investigate the occurrence of oseltamivir and oseltamivir carboxylate in surface waters in the course of a swine flu pandemic, ii) to assess whether surface water concentrations of these compounds can be used to follow the course of a swine flu epidemic in surface waters and iii) to assess the occurrence of other antiviral drugs such as acyclovir in surface waters over a longer time period.

3.2 MATERIALS AND METHODS

3.2.1 SAMPLING OF SURFACE WATER

Daily composite samples (24 h) from the river Rhine near Koblenz were taken during a period of 43 days (1st November – 13th December). The period during which the most swine flu cases were reported which is in the following termed as the flu epidemic was approx. between 3rd November and 3rd December. Thus the first and the last samples were taken before and after the flu epidemic, respectively. The sampling device was cooled (4°C) to prevent microbial degradation during storage. Maximum storage time was 7 days until analysis.

3.2.2 ANALYTICAL METHOD

For the extraction of antiviral drugs ENV+ 500 mg (Biotage, Uppsala, Sweden) SPE cartridges were used. Details on the SPE method and the LC-tandem MS analysis can be found in Prasse et al.[1] Briefly, samples (500 mL) were adjusted to pH 8 (ammonium

hydroxide (25%)) before the internal standards were added. After the extraction, cartridges were dried under a gentle stream of nitrogen and analytes were eluted with 5×2mL methanol/acetone 50/50+0.2% formic acid (v/v). Sample extracts were evaporated to 100 µL under a gentle stream of nitrogen and reconstituted to 1 mL with the aqueous buffer (5 mM NH_4-formate) before analysis via LC-tandem MS.

3.3 Results and Discussion

3.3.1 Concentrations of oseltamivir and oseltamivir carboxylate in the Rhine

Concentrations of oseltamivir and oseltamivir carboxylate in the surface water samples from the Rhine ranged from 8 to 48 ng L^{-1} and 1.4 to 20 ng L^{-1}, respectively. Highest concentrations of oseltamivir were detected at the beginning of the sampling campaign (maximum on 1st November), whereas for oseltamivir carboxylate highest concentrations were observed significantly later (maximum on 22nd November; Fig. 1). This is surprising considering the fact that both compounds are excreted simultaneously from treated individuals as oseltamivir is metabolized by hepatic esterases to oseltamivir carboxylate.[12, 13] However, for the river Rhine it has been shown that oseltamivir can also be derived from industrial discharges of a manufacturer.[1] The allocation to different sources is possible as it has been shown that the oseltamivir to oseltamivir carboxylate ratio (OP/OC ratio)) differs substantially among both sources (human excretions: OP/OC ~ 0.2; industrial discharges: OP/OC ~ 13). In surface waters which only receive treated municipal wastewater a mean OP/OC ratio of approx. 1.1 is obtained. As both oseltamivir and oseltamivir carboxylate have been shown to be persistent in the aquatic environment with half-lives of >59 days[6, 7] the OP/OC ratio can be used to assess individual source contributions. Based on these findings, the elevated OP/OC ratios in the beginning of the sampling period indicate the dominance of industrial discharges. In contrast, significantly lower ratios during the period from 9th - 29th November, ranging only from 1.1 to 2.2 (mean 1.7), point towards the dominance of OP and OC derived from human excretions, whereas increasing ratios at the end of the sampling period (30th November - 13th December) indicating the contribution of both sources.

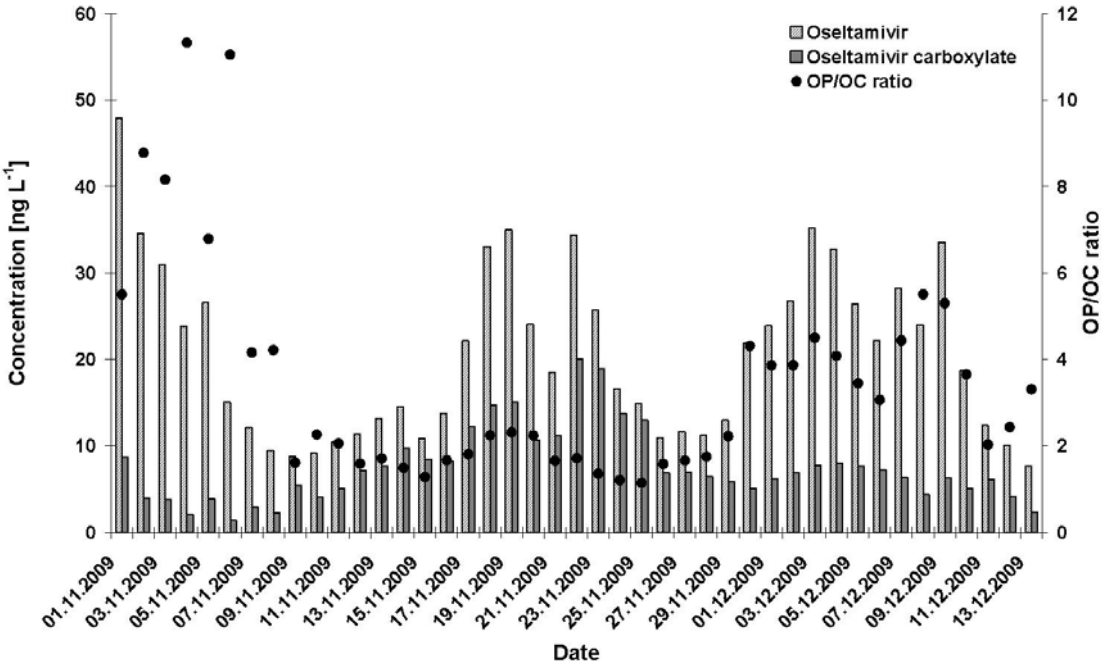

FIGURE 1. Concentrations of oseltamivir (OP) and oseltamivir carboxylate (OC) in 24 h composite samples taken in the Rhine river during the swine flu pandemic in November/December 2009. In addition, calculated ratios of oseltamivir/oseltamivir carboxylate (OP/OC ratio) are given.

3.3.2 LOADS OF OSELTAMIVIR AND OSELTAMIVIR CARBOXYLATE IN THE RHINE

Even though the calculation of OP/OC ratios is independent of the river discharge, the calculation of river loads is crucial for a detailed data interpretation.[14, 15] In particular, decreasing concentrations of a compound in rivers can either indicate a reduced emission, e.g. of WWTPs, but can also be caused by dilutional effects, e.g. due to higher river discharges. Variations in river discharges are most pronounced in small rivers but also for the Rhine large variations were observed with the discharge varying between 800 $m^3 s^{-1}$ and 2500 $m^3 s^{-1}$ during the sampling period (Fig. 3). Furthermore, the calculation of river loads enables for an assessment of the number of treated individuals in a river catchment.[16-18] As shown in Fig. 2, river loads of oseltamivir and oseltamivir carboxylate during the sampling period ranged between 0.9 and 4.6 kg d^{-1} as well as 0.1 and 2.2 kg d^{-1}, respectively. For oseltamivir highest loads (> 2 kg d^{-1}) were observed in the beginning (1st - 5th November), in the middle (17th - 23rd November) and at the end of the sampling period (30th November - 12th December). The highest river loads of oseltamivir carboxylate were observed between 17th and 25th November with a more than fourfold increase of river loads till 22nd November compared to the beginning of the sampling period, indicating the widespread use of oseltamivir for the treatment of (swine) influenza. This is in good agreement with the total number of swine flu cases in Germany[19] indicating that river loads of oseltamivir can be used to follow the course of a swine flu epidemic in surface waters (Fig. 2).

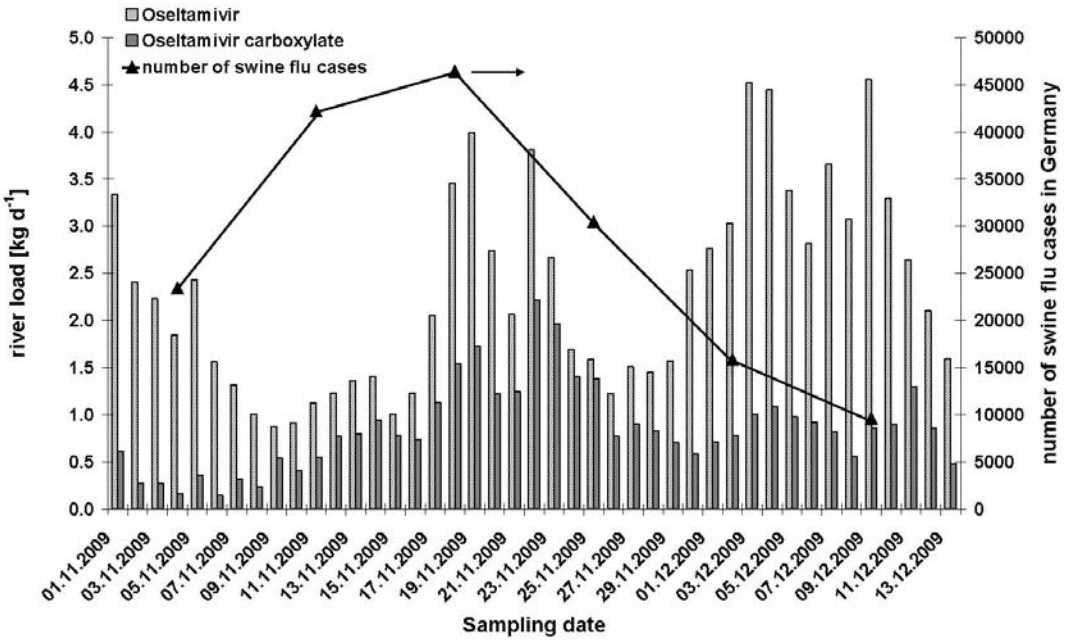

FIGURE 2. River loads of oseltamivir and its active metabolite oseltamivir carboxylate in the Rhine river near Koblenz (Germany) during the swine flu pandemic in November/December 2009. Loads were calculated based on daily mean river discharge data. In addition, the number of weekly reported swine flu cases in Germany is given.[19]

3.3.3 ESTIMATED NUMBER OF TREATED INDIVIDUALS BASED ON RIVER LOADS

The influence of industrial discharges to the river load of especially oseltamivir in the Rhine makes an estimation of the number of treated individuals difficult and the results should be interpreted with care. Nevertheless, as for industrial discharges the proportion of oseltamivir carboxylate is low (~1/13 corresponding to approx. 8%)[1] the river loads of oseltamivir carboxylate can be used to estimate the amount of oseltamivir derived from human excretions based on OP/OC ratios of a large number (n = 48) of samples taken from surface waters which do not receive any industrial discharges of these compounds (mean OP/OC ratio of 1.1).[1] This thereby allows to estimate the total amount of oseltamivir consumed by treated individuals (sum of oseltamivir carboxylate and oseltamivir derived from human excretions). Finally, the number of treated individuals in the river catchment can be calculated using the defined daily dose (DDD) of oseltamivir (150 mg).[20] The obtained results were compared to the reported number of swine flu cases in the river catchment (Fig. 3). The number of infected individuals was thereby calculated from the total population living in the catchment area (28 Mio. people for the Rhine upstream of Koblenz; Dr. M. Keller (BfG, Koblenz, personal communication)), assuming that the proportion of swine flu cases in relation to the total population in the whole Rhine catchment (besides Germany primarily Switzerland) was similar to those reported for Germany. Due to the high uncertainties associated with the calculation, the results should therefore not be

taken as absolute values but rather as an indication and as can be seen in Fig. 3 a good agreement was observed.

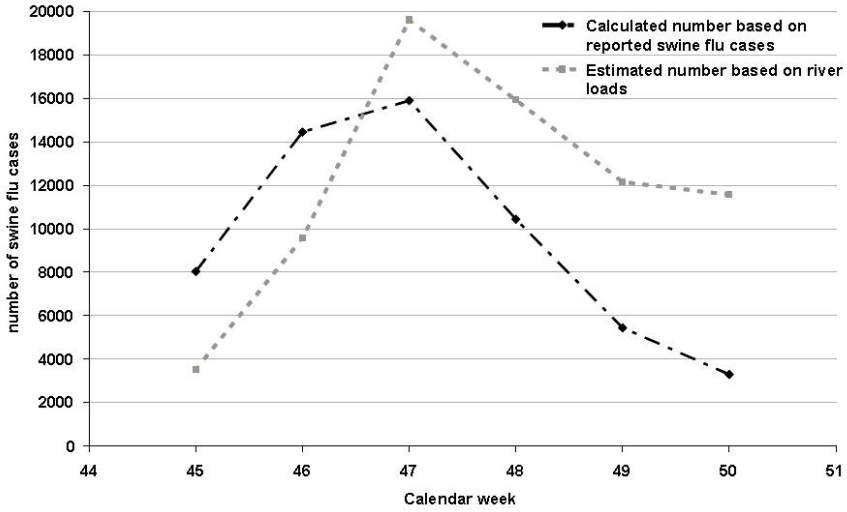

FIGURE 3. Comparison of calculated and estimated numbers of swine flu cases in the Rhine catchment upstream of Koblenz. Calculated numbers are based on the total population living in the Rhine catchment and the proportion of swine flu cases on the total population in Germany. Estimated numbers are based on river loads of oseltamivir and oseltamivir carboxylate in the Rhine derived from human excretions (no industrial discharges).

3.3.4 OCCURRENCE OF ACYCLOVIR AND ZIDOVUDINE IN THE RIVER RHINE

Besides oseltamivir and oseltamivir carboxylate also acyclovir and zidovudine were detected in the samples taken from the river Rhine. As can be seen in Fig. 4 concentrations of zidovudine were relatively homogenous throughout the sampling period, varying between 3.3 and 8.2 ng L^{-1} (average concentration: 5.9 ng L^{-1}; n = 43), whereas for acyclovir larger variations were observed with concentrations ranging from < LOQ to 7.4 ng L^{-1} (average concentration: 3.7 ng L^{-1}; n = 40). This is in good agreement with results from Prasse et al., who observed concentrations of acyclovir and zidovudine in the Rhine in summer 2009 up to 3.6 and 5.1 ng L^{-1}, respectively.[1]

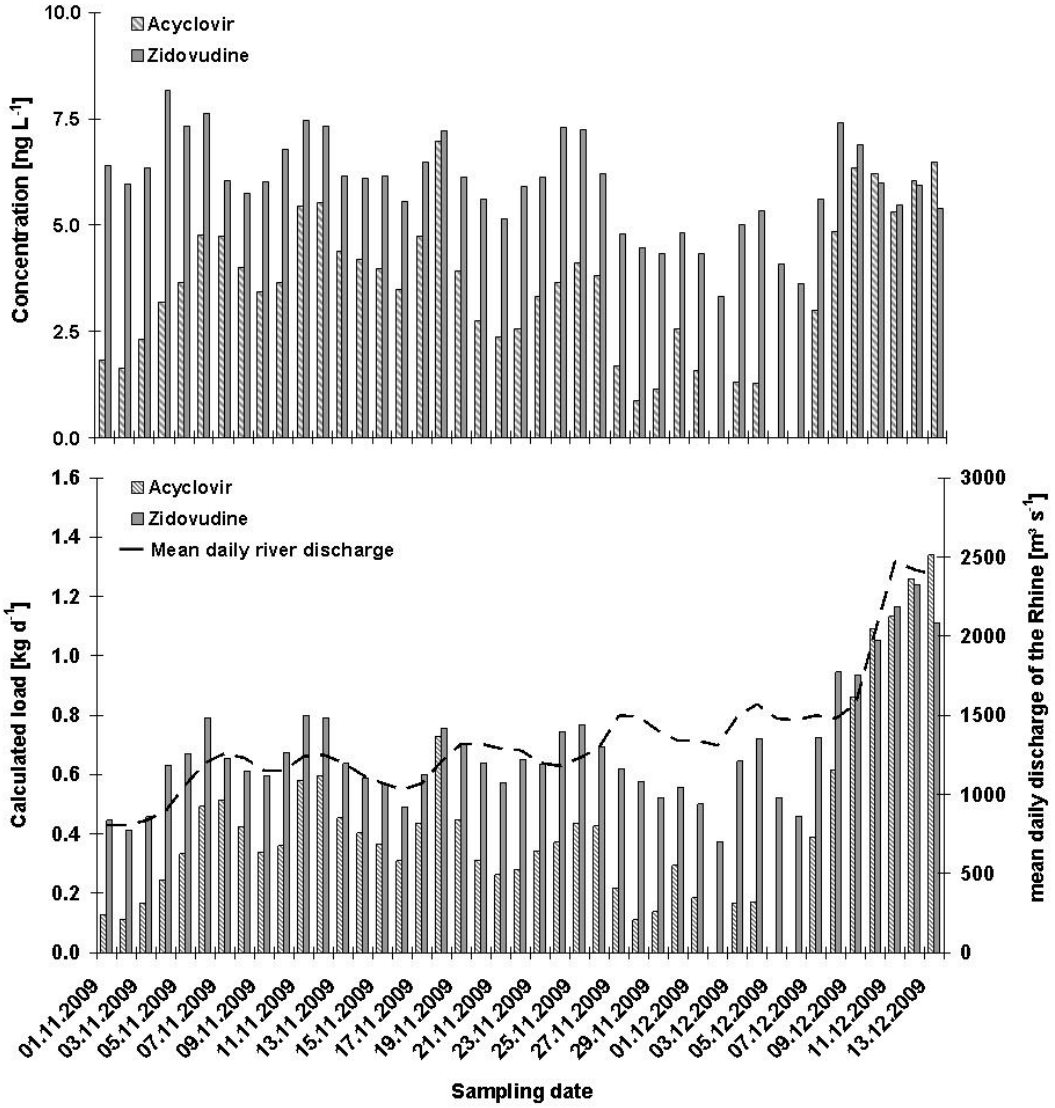

FIGURE 4. Concentrations (top) and calculated river loads (bottom, together with mean daily river discharge data) of acyclovir and zidovudine in the river Rhine in November and December 2009.

The calculation of river loads revealed large variations with a maximum for both compounds at the end of the sampling period up to 1.3 and 1.2 kg d^{-1} for acyclovir and penciclovir, respectively (Fig. 4). In general, river loads can be expected to vary significantly for pharmaceuticals which are not constantly used throughout the week such as X-ray contrast media, whereas for others river loads can be expected to be constant.[21] Even though the latter can in general also be expected for antiviral drugs such as acyclovir and zidovudine, river loads of both compounds varied significantly throughout the sampling period (Fig. 4). As can be seen in Fig. 4 a good correlation between river loads and mean daily river discharges was observed. This is especially true at the end of the monitoring campaign with both the river discharge (from approx. 1500 to 2500 m^3 s^{-1}) and the river loads of acyclovir (from 0.2 to 1.2 kg d^{-1}) and zidovudine (from 0.5 to 1.2 kg d^{-1}) increasing significantly. Therefore, it is indicated

that the observed variations are caused by limited WWTP treatment capabilities and combined sewer overflows (CSO) during rain events.[22-24] This is especially true for combined sewer systems in which rainwater runoff, domestic sewage and industrial wastewater are collected in the same pipe. During periods of heavy rainfall, however, the wastewater volume can exceed the treatment capacities of WWTPs with the consequence that the untreated wastewater is directly discharged into the receiving waters.[14, 25, 26] For example, Jonkers et al. showed that increasing mass flows of endocrine disruptors such as bisphenol A in the Glatt river catchment in Switzerland during rain events were caused by discharges of untreated wastewater.[27] Considering that acyclovir is extensively removed in conventional WWTP (>98%)[1] whereas the removal efficiency of zidovudine is significantly lower (0-68%), the more pronounced increase of river loads of acyclovir compared to zidovudine during rain events can be explained by the discharge of untreated wastewater into receiving waters.

In summary, this study revealed that swine flu pandemics can be followed by the analysis of anti-influenza drugs in surface waters with a good correlation between swine flu cases and observed river loads. Furthermore, it was shown that also discharges from the pharmaceutical industry can contribute significantly to the presence of pharmaceuticals in the aquatic environment. Finally, elevated river loads during higher discharge events indicate the influence of wastewater which is discharged untreated into receiving waters.

3.4 REFERENCES

(1) Prasse, C.; Schluesener, M. P.; Schulz, R.; Ternes, T. A., Antiviral Drugs in Wastewater and Surface Waters: A New Pharmaceutical Class of Environmental Relevance? Environmental Science & Technology 2010, 44 (5), 1728-1735.

(2) Singer, A. C.; Colizza, V.; Schmitt, H.; Andrews, J.; Balcan, D.; Huang, W. E.; Keller, V. D. J.; Vespignani, A.; Williams, R. J., Assessing the Ecotoxicologic Hazards of a Pandemic Influenza Medical Response. Environmental Health Perspectives 2011, 119 (8), 1084-1090.

(3) Singer, A. C.; Howard, B. M.; Johnson, A. C.; Knowles, C. J.; Jackman, S.; Accinelli, C.; Caracciolo, A. B.; Bernard, I.; Bird, S.; Boucard, T., et al., Meeting Report: Risk Assessment of Tamiflu Use Under Pandemic Conditions. Environmental Health Perspectives 2008, 116 (11), 1563-1567.

(4) Fick, J.; Lindberg, R. H.; Tysklind, M.; Haemig, P. D.; Waldenstrom, J.; Wallensten, A.; Olsen, B., Antiviral Oseltamivir Is not Removed or Degraded in Normal Sewage Water Treatment: Implications for Development of Resistance by Influenza A Virus. Plos One 2007, 2 (10).

(5) Ghosh, G. C.; Nakada, N.; Yamashita, N.; Tanaka, H., Occurrence and fate of oseltamivir carboxylate (Tamiflu) and amantadine in sewage treatment plants. Chemosphere 2010, 81 (1), 13-17.

(6) Accinelli, C.; Sacca, M. L.; Fick, J.; Mencarelli, M.; Lindberg, R.; Olsen, B., Dissipation and removal of oseltamivir (Tamiflu) in different aquatic environments. Chemosphere 2010, 79 (8), 891-897.

(7) Bartels, P.; von Tuempling, W., Jr., The environmental fate of the antiviral drug oseltamivir carboxylate in different waters. Science of the Total Environment 2008, 405 (1-3), 215-225.

(8) Goncalves, C.; Perez, S.; Osorio, V.; Petrovic, M.; Alpendurada, M. F.; Barcelo, D., Photofate of Oseltamivir (Tamiflu) and Oseltamivir Carboxylate under Natural and Simulated Solar Irradiation: Kinetics, Identification of the Transformation Products, and Environmental Occurrence. Environmental Science & Technology 2011, 45 (10), 4307-4314.

(9) Tong, A. Y. C.; Braund, R.; Tan, E. W.; Tremblay, L. A.; Stringer, T.; Trought, K.; Peake, B. M., UV-induced photodegradation of oseltamivir (Tamiflu) in water. Environmental Chemistry 2011, 8 (2), 182-189.

(10) Ghosh, G. C.; Nakada, N.; Yamashita, N.; Tanaka, H., Oseltamivir Carboxylate, the Active Metabolite of Oseltamivir Phosphate (Tamiflu), Detected in Sewage Discharge and River Water in Japan. Environmental Health Perspectives 2010, 118 (1), 103-107.

(11) Soderstrom, H.; Jarhult, J. D.; Olsen, B.; Lindberg, R. H.; Tanaka, H.; Fick, J., Detection of the Antiviral Drug Oseltamivir in Aquatic Environments. Plos One 2009, 4 (6).

(12) Davies, B. E., Pharmacokinetics of oseltamivir: an oral antiviral for the treatment and prophylaxis of influenza in diverse populations. Journal of Antimicrobial Chemotherapy 2010, 65, II5-II10.

(13) He, G.; Massarella, J.; Ward, P., Clinical pharmacokinetics of the prodrug oseltamivir and its active metabolite Ro 64-0802. Clinical Pharmacokinetics 1999, 37 (6), 471-484.

(14) Phillips, P.; Chalmers, A., Wastewater Effluent, Combined Sewer Overflows, and Other Sources of Organic Compounds to Lake Champlain. Journal of the American Water Resources Association 2009, 45 (1), 45-57.

(15) Shala, L.; Foster, G. D., Surface Water Concentrations and Loading Budgets of Pharmaceuticals and Other Domestic-Use Chemicals in an Urban Watershed (Washington, DC, USA). Archives of Environmental Contamination and Toxicology 2010, 58 (3), 551-561.

(16) Irvine, R. J.; Kostakis, C.; Felgate, P. D.; Jaehne, E. J.; Chen, C.; White, J. M., Population drug use in Australia: A wastewater analysis. Forensic Science International 2011, 210 (1-3), 69-73.

(17) Postigo, C.; Lopez de Alda, M.; Barcelo, D., Evaluation of drugs of abuse use and trends in a prison through wastewater analysis. Environment International 2011, 37 (1), 49-55.

(18) van Nuijs, A. L. N.; Mougel, J.-F.; Tarcomnicu, I.; Bervoets, L.; Blust, R.; Jorens, P. G.; Neels, H.; Covaci, A., Sewage epidemiology - A real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium. Environment International 2011, 37 (3), 612-621.

(19) Robert Koch Institute; (www.rki.de/influenza) RKI Influenza. accessed 15.03.2010.

(20) WHO Collaborating Centre for Drug Statistics Methodology; (http://www.whocc.no/atc_ddd_index/) WHO ATC/DDD index. accessed 23.06.2011.

(21) Drewes, J. E.; Fox, P.; Jekel, M., Occurrence of iodinated X-ray contrast media in domestic effluents and their fate during indirect potable reuse. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering 2001, 36 (9), 1633-1645.

(22) Ahnert, M.; Traenckner, J.; Guenther, N.; Hoeft, S.; Krebs, P., Model-based comparison of two ways to enhance WWTP capacity under stormwater conditions. Water Science and Technology 2009, 60 (7), 1875-1883.

(23) Benotti, M. J.; Brownawell, B. J., Distributions of pharmaceuticals in an urban estuary during both dry- and wet-weather conditions. Environmental Science & Technology 2007, 41 (16), 5795-5802.

(24) Wilen, B. M.; Lumley, D.; Mattsson, A.; Mino, T., Rain events and their effect on effluent quality studied at a full scale activated sludge treatment plant. Water Science and Technology 2006, 54 (10), 201-208.

(25) Buerge, I. J.; Poiger, T.; Muller, M. D.; Buser, H. R., Combined sewer overflows to surface waters detected by the anthropogenic marker caffeine. Environmental Science & Technology 2006, 40 (13), 4096-4102.

(26) Weyrauch, P.; Matzinger, A.; Pawlowsky-Reusing, E.; Plume, S.; von Seggern, D.; Heinzmann, B.; Schroeder, K.; Rouault, P., Contribution of combined sewer overflows to trace contaminant loads in urban streams. Water Research 2010, 44 (15), 4451-4462.

(27) Jonkers, N.; Kohler, H.-P. E.; Dammshaeuser, A.; Giger, W., Mass flows of endocrine disruptors in the Glatt River during varying weather conditions. Environmental Pollution 2009, 157 (3), 714-723.

4

BIOTRANSFORMATION OF THE ANTIVIRAL DRUGS ACYCLOVIR AND PENCICLOVIR IN ACTIVATED SLUDGE TREATMENT

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Abstract

The biotransformation of the two antiviral drugs, acyclovir (ACV) and penciclovir (PCV), was investigated in contact with activated sludge. Biodegradation kinetics were determined, and transformation products (TPs) were identified using Hybrid Linear Ion Trap- FT Mass Spectrometry (LTQ Orbitrap Velos) and 1D (^{1}H NMR, ^{13}C NMR) and 2D (^{1}H,^{1}H-COSY, ^{1}H-^{13}C-HSQC) NMR Spectroscopy. ACV and PCV rapidly dissipated in the activated sludge batch systems with half-lives of 5.3 and 3.4 h and first-order rate constants in relation to the amount of suspended solids (SS) of 4.9 ± 0.1 L g_{ss}^{-1} d^{-1} and 7.6 ± 0.3 L g_{ss}^{-1} d^{-1}, respectively. For ACV only a single TP was found, whereas eight TPs were identified for PCV. Structural elucidation of TPs exhibited that transformation only took place at the side chain leaving the guanine moiety unaltered. The oxidation of the primary hydroxyl group in ACV resulted in the formation of carboxy-acyclovir (Carboxy-ACV). For PCV, transformation was more diverse with several enzymatic reactions taking place such as the oxidation of terminal hydroxyl groups and β-oxidation followed by acetate cleavage. Analysis of different environmental samples revealed the presence of Carboxy-ACV in surface and drinking water with concentrations up to 3200 ng L^{-1} and 40 ng L^{-1}, respectively.

4.1 INTRODUCTION

Pharmaceutical active substances are ubiquitously present in the environment. Antiviral drugs are a class of pharmaceuticals which have recently been detected in wastewater treatment plant (WWTP) effluents and surface waters.[1-3] Acyclovir and penciclovir are used for the treatment of herpes infections[4] and have shown to be extensively eliminated in conventional wastewater treatment with removal rates exceeding 95%.[3] However, up to now no information is available whether these substances are mineralized or only partially transformed in WWTPs. In the past few years, transformation products (TPs) of environmental micropollutants have attracted a broad scientific interest as evidence is increasing that most pollutants are not completely removed in wastewater treatment (i.e. mineralized) but are often only slightly modified,[5-7] resulting in TPs with a comparable or increased toxicity compared to parent compounds.[8-10] The elucidation of the chemical structure of TPs is time consuming as sufficient quantities of individual TPs have to be isolated to carry out NMR or IR spectroscopy. Recently, several studies have been published proposing the use of high resolution mass spectrometry (HR-MS) in combination with biotransformation pathway prediction models for the identification of unknown TPs in batch experiments and for the screening of TPs in environmental samples such as wastewater.[11,12] The objectives of this study were to investigate the biotransformation of ACV and PCV in activated sludge, to identify TPs and to elucidate microbial biotransformation pathways. Furthermore, TPs observed in batch experiments were compared to those predicted by the University of Minnesota pathway prediction system (UM-PPS).[13] Finally, LC tandem mass spectrometry was used to determine whether the TPs of acyclovir and penciclovir are found in aqueous environmental samples.

4.2 MATERIALS AND METHODS

4.2.1 CHEMICALS AND STANDARDS

Details on the used chemicals and standards are provided in the Supporting Information (SI).

4.2.2 TRANSFORMATION KINETICS OF ACV AND PCV IN ACTIVATED SLUDGE

For the kinetic experiments secondary sewage sludge from a municipal WWTP (WWTP1; see SI for further details) was taken as a grab sample from the nitrification zone of the activated sludge unit. Activated sludge was transported to the laboratory, and the batch experiments were launched on the same day. The sludge (200 mL; total suspended solids (TSS): 4 g_{SS} L^{-1}, total organic carbon (TOC): 0.3 g g_{SS}^{-1}) was poured into 1 L amber glass bottles and filled up with 800 mL of WWTP effluent (final sludge concentration: 0.8 g_{SS} L^{-1}). Sludge was diluted to minimize sorption of ACV and PCV as well as to increase the temporal resolution of biodegradation kinetics. Test vessels were continuously stirred and aerated to guarantee aerobic conditions. To maintain a constant pH (pH 7 ± 0.1) during the experiments, CO_2 was mixed into the air flow at a definite ratio (~1:20) using a rotameter. After an equilibration period of 1 h, ACV and PCV were spiked individually into the test vessels attaining an initial concentration of 4 µg L^{-1}. The test duration was 80 h, and the temperature was 22°C ± 2°C. At each sampling time point, 50 mL were taken, acidified to inhibit microbial activity (pH 3, 3.5 M H_2SO_4), filtered (0.45 µm cellulose nitrate filters), and stored in the freezer (-25°C) until analysis. Samples were extracted using SPE as described in Prasse et al.[3] Briefly, pH was adjusted to pH 8 using NH_4OH (25%) prior to the addition of deuterated internal standards (acyclovir-d4 and penciclovir-d4). Samples were applied on Isolute ENV^+ cartridges (500 mg sorbent, Biotage, Sweden) which were eluted using 5 x 2 mL methanol/acetone (50/50, v/v) + 0.2% formic acid. Extracts were evaporated to 50 µL under a gentle stream of nitrogen. Finally, sample extracts were filled up to 1 mL with 5 mM NH_4-formate buffer prior to LC-tandem MS (LC-QqQ) analysis.

4.2.3 AQUEOUS ENVIRONMENTAL SAMPLES

Composite samples (24 h, WWTP1) and grab samples (WWTP2) of influent and effluent were taken during a dry weather period (May 2010). Further information about the WWTP characteristics and the treatment processes applied are given in the SI. Surface water (grab samples) was taken from different rivers and streams in Germany with various compositions of treated wastewater (<10 to >90%). Concentrations of PCV TPs were below the limit of quantification (LOQ; Table S3 in the SI) in all environmental samples due to the low concentrations of PCV in raw waste water (<50 ng L^{-1}).[3] Therefore, Carboxy-ACV was the only TP quantitatively analyzed. To elucidate the environmental fate of Carboxy-ACV from surface water to finished drinking water

several samples were taken from the effluent of a WWTP (WWTP3), a reservoir and finished drinking water. Additionally, groundwater was sampled from two wells with oxic (approximately 11 m depth) and anoxic conditions (approximately 38 m depth). The drinking water treatment plant (DWTP) utilizes bank filtration, groundwater passage, and slow sand filtration. All samples (grab samples and 24 h composite samples) were taken on the same day, acidified to pH 3 using 3.5 M H_2SO_4 (1 mL L^{-1}) directly after sampling, transported to the laboratory and extracted within 72 h. To distinguish whether concentration variations are attributable to dilution or biodegradation, acesulfame, an artificial sweetener used as wastewater indicator due to its environmental recalcitrance, was analyzed in all samples.[14,15] Due to the high polarity of Carboxy-ACV, sorption could be ruled out. Details on the analysis of acesulfame are given in the SI.

4.2.4 Isolation of TPs

For isolation of TPs from the batch experiments, acyclovir and penciclovir were spiked to attain an initial concentration of 150 mg L^{-1}. The experimental setup was identical to the kinetic experiments. The sludge (4 g_{SS} L^{-1}) was diluted with pristine groundwater (1:16) in 500 mL amber glass bottles (final sludge concentration: 0.25 g_{SS} L^{-1}). The total test duration was 29 d. At the end of the incubation, the entire test volumes were filtered through 0.45 µm cellulose nitrate filters, immediately frozen (-25°C), and lyophilized. Details on the semipreparative HPLC/UV system used to fractionate the TPs are provided in the SI. An aliquot of each fraction was analyzed by LC-tandemMS (API-4000 Q Trap; Applied Biosystems/ MDS Sciex, Darmstadt, Germany) to confirm the purity of the isolated substances. Finally, each fraction was lyophilized to evaporate the solvents and the remaining water. The attained solid material of the TPs was used for NMR measurements and for the preparation of stock solutions for quantitative analyses. Stock solutions and working standards (2 µg mL^{-1}) of individual TPs (Carboxy-ACV, PCV TP209, PCV TP233, PCV TP237; PCV TP249A, PCV TP249B; PCV TP251; PCV TP267; PCV TP281) were prepared in Milli-Q-H2O/MeOH (50/50, v/v) .

4.2.5 Identification of TPs via HR-MS and HR-MS^N Experiments

Accurate MS and MS/MS analyses of parent compounds and their microbial transformation products were performed using a Hybrid Linear Ion Trap-Orbitrap Mass Spectrometer (LTQ Orbitrap Velos, Thermo Scientific, Bremen, Germany) by direct injection using a syringe pump (flow rate: 8 µL min^{-1}) in ESI positive ion mode. To screen samples from batch experiments directly for TPs without isolation via semipreparative HPLC, the LTQ Orbitrap Velos was coupled to a Thermo Scientific Accela liquid chromatography system (Accela pump and autosampler). Further information on the applied setup, chromatographic conditions, and the data-dependent acquisition parameters are given in the SI. The LTQ Orbitrap Velos was used for all experiments to obtain exact masses of parent and fragment ions. External

calibration was performed prior to the analysis of each batch to ensure accurate mass determinations with a resolution of 60,000. A mixture of n-butylamine, caffeine, and Ultramark 1621 (mixture of fluorinated phosphazines) was used as mass calibrant and accuracy always was within 0.5 ppm.

4.2.6 Identification of TPs via NMR

Approximately 10 mg of acyclovir and penciclovir, and 0.5 - 8 mg of the respective TPs, isolated from batch experiments with activated sludge, were dissolved in 0.8 mL of DMSO-d6. NMR analyses were carried out on a Bruker DRX Advance 500 and a Advance 700 instrument (Rheinstetten, Germany). ^{1}H NMR spectra were recorded at 500 and 700 MHz, ^{13}C NMR spectra at 125 and 176 MHz, respectively. Homo- and heteronuclear chemical shift correlations were determined by ^{1}H,^{1}H-COSY and ^{1}H,^{13}C-HSQC. All spectra and correlation experiments were performed at 298.3 K, and chemical shifts were presented in ppm referenced to tetramethylsilane (TMS). Further details are provided in the SI.

4.2.7 Mass Balances in Batch Experiments

For determination of mass balances during batch experiments, a sludge concentration of 0.25 g_{SS} L^{-1} and initial ACV and PCV concentration of 20 mg L^{-1} were used. The relatively high dilution of the sludge and high analyte concentrations were selected to allow the simultaneous analysis of dissolved organic carbon (DOC) content. A HPLC system, coupled to a fluorescence detector, was more sensitive and not as influenced by background noise than UV-detection and therefore used to determine the mass balances. Excitation wavelength was set to 260 nm and emission wavelength to 360 nm. Sensitivity in fluorescence detection is assumed to be equivalent for parent compounds and evolving TPs because the and remained unaltered during acyclovir and penciclovir biodegradation. DOC analysis was performed according to DIN EN 1484 after acidification to pH 2.1 (30% HCl) and filtration (0.45 µm; cellulose nitrate filter). This allowed to determine the importance of i) irreversible sorption and/or incorporation into the microbial biomass, ii) mineralization, and iii) TPs which were not amendable to MS and UV-/fluorescence-detection and/or eluted with the dead time on the used HPLC columns.

4.2.8 Elucidation of Microbial Transformation Pathway of PCV

For elucidation of the microbial transformation pathway of PCV, individual TPs were spiked at a concentration of 15 µg L^{-1} into sewage sludge (0.8 g_{SS} L^{-1}) and analyzed via HR-MS. Blanks (autoclaved groundwater) were run in parallel to exclude abiotic (i.e., chemical) processes causing TP formation.

4.2.9 Analysis of ACV, PCV, and their TPs in Aqueous Environmental Samples.

For all TPs observed in batch experiments an analytical method was developed applying SPE followed by LC ESI-tandem MS detection. A detailed description of the SPE procedure, the LC-tandem MS analysis, the method validation, and the quantification of TPs in aqueous samples can be found in the SI. Briefly, aqueous environmental samples (100 - 500 mL) were extracted using Isolute ENVþ cartridges (6 mL, 500 mg, Biotage, Uppsala, Sweden) under acidic conditions (pH 2.5 and pH 4) after the addition of acyclovir-d4 and penciclovir-d4 as surrogate standards. For LC-ESI-tandem MS analysis two MRM transitions were used for quantification and confirmation of TPs in aqueous environmental samples. Analysis of ACV and PCV was performed as described above for the kinetic batch experiments.

TABLE 1. Accurate mass measurements of PCV and its TPs using LTQ Orbitrap Velos Electro-spray Ionization in positive ion mode.

	Formula	Measd. mass	Calc. mass	mass error [ppm]	RDB[a]	Loss
PCV						
[M+H]+	$C_{10}H_{16}O_3N_5$	254.1242	254.1248	-2.23	5.5	
m/z 236	$C_{10}H_{14}O_2N_5$	236.1136	236.1142	-2.42	6.5	**-H_2O**
m/z 152	$C_5H_6ON_5$	152.0564	152.0567	-2.21	5.5	
m/z 135	$C_5H_3ON_4$	135.0299	135.0301	-1.91	6.5	
		PCV TP281				
[M+H]+	$C_{10}H_{12}O_5N_5$	282.0828	282.0833	-1.83	7.5	
m/z 238	$C_9H_{12}O_3N_5$	238.0931	238.0935	-1.75	6.5	**-CO_2**
m/z 220	$C_9H_{10}O_2N_5$	220.0825	220.0829	-1.82	7.5	**-CO_2, -H_2O**
m/z 194	$C_8H_{12}ON_5$	194.1033	194.1036	-1.79	5.5	**-CO_2, -CO_2**
m/z 152	$C_5H_6ON_5$	152.0564	152.0567	-1.69	5.5	
m/z 135	$C_5H_3ON_4$	135.0300	135.0301	-1.31	6.5	
		PCV TP249A				
[M+H]+	$C_{10}H_{12}O_3N_5$	250.0931	250.0935	-1.62	7.5	
m/z 222	$C_9H_{12}O_2N_5$	222.0980	222.0986	-2.66	6.5	**-CO**
m/z 152	$C_5H_6ON_5$	152.0563	152.0567	-2.28	5.5	
m/z 135	$C_5H_3ON_4$	135.0299	135.0301	-2.13	6.5	
		PCV TP249B				
[M+H]+	$C_{10}H_{12}O_3N_5$	250.0931	250.0935	-1.46	7.5	
m/z 232	$C_{10}H_{10}O_2N_5$	232.0826	232.0829	-1.30	8.5	**-H_2O**
m/z 152	$C_5H_6ON_5$	152.0564	152.0567	-1.69	5.5	
m/z 135	$C_5H_3ON_4$	135.0300	135.0301	-1.39	6.5	
		PCV TP267				
[M+H]+	$C_{10}H_{14}O_4N_5$	268.1036	268.1040	-1.79	6.5	
m/z 250	$C_{10}H_{12}O_3N_5$	250.0932	250.0935	-1.14	7.5	**-H_2O**
m/z 232	$C_{10}H_{10}O_2N_5$	232.0824	232.0829	-1.99	8.5	**-H_2O, -H_2O**
m/z 152	$C_5H_6ON_5$	152.0564	152.0567	-2.08	5.5	
m/z 135	$C_5H_3ON_4$	135.0299	135.0301	-2.05	6.5	
		PCV TP237				
[M+H]+	$C_9H_{12}O_3N_5$	238.0930	238.0935	-1.87	6.5	
m/z 220	$C_9H_{10}O_2N_5$	220.0824	220.0829	-2.10	7.5	**-H_2O**
m/z 152	$C_5H_6ON_5$	152.0564	152.0567	-2.15	5.5	
m/z 135	$C_5H_3ON_4$	135.0299	135.0301	-2.05	6.5	
		PCV TP233				
[M+H]+	$C_{10}H_{12}O_2N_5$	234.0981	234.0986	-2.01	7.5	
m/z 216	$C_{10}H_{10}ON_5$	216.0876	216.0880	-1.97	8.5	**-H_2O**
m/z 152	$C_5H_6ON_5$	152.0563	152.0567	-0.36	5.5	
m/z 135	$C_5H_3ON_4$	135.0299	135.0301	-1.91	6.5	
m/z 83	$C_3H_5N_3$	83.0487	83.0478	-2.02	5.5	
		PCV TP209				
[M+H]+	$C_7H_8O_3N_5$	210.0616	210.0622	-2.50	6.5	
m/z 166	$C_6H_8ON_5$	166.0719	166.0723	-2.51	5.5	**-CO_2**
m/z 164	$C_6H_6ON_5$	164.0563	164.0567	-2.36	6.5	**-HCOOH**
m/z 152	$C_5H_6ON_5$	152.0563	152.0567	-2.48	5.5	
m/z 135	$C_5H_3ON_4$	135.0299	135.0301	-1.91	6.5	
		PCV TP251				
[M+H]+	$C_{10}H_{14}O_3N_5$	252.1086	252.1091	-2.05	6.5	
m/z 234	$C_{10}H_{12}O_2N_5$	234.0981	234.0986	-2.01	7.5	**-H_2O**
m/z 152	$C_5H_6ON_5$	152.0564	152.0567	-2.02	5.5	
m/z 135	$C_5H_3ON_4$	135.0299	135.0301	-1.98	6.5	

[a]RDB Ring and double-bond equivalents

4.3 RESULTS AND DISCUSSION

4.3.1 TRANSFORMATION OF ACV AND PCV IN ACTIVATED SLUDGE

Complete transformation of ACV and PCV was observed in the activated sludge systems after 24 h. For both antiviral drugs an exponential decrease was observed, indicating pseudo first order degradation kinetics (SI Figure S1). Therefore, transformation rate constants k_{biol} were calculated using

$$k_{biol} = -\frac{\ln\left(\frac{C_t}{C_0}\right)}{t \cdot X_{SS}} \qquad t_{1/2} = \frac{\ln(2)}{k_{biol} \cdot X_{SS}}$$

where C is the total compound concentration [ng L^{-1}], t is time [d], k_{biol} is the reaction rate constant [L g_{SS}^{-1} d^{-1}], X_{SS} is the suspended sludge concentration in the batch system [g_{SS} L^{-1}], and $t_{1/2}$ is the degradation half-life [d].

The 'first order' refers to the direct proportionality of the transformation rate to the soluble substance concentration. The term 'pseudo' refers to its proportionality to the sludge concentration X_{SS}, which can be assumed to be constant for short-term batch experiments.[16] For acyclovir and penciclovir the calculated degradation half-lives (time period for 50% transformation of initially added substance) were 5.3 and 3.4 h. The degradation rate constants of 4.9 ± 0.1 L g_{SS}^{-1} d^{-1} (r^2 = 0.999) and 7.6 ± 0.3 L g_{SS}^{-1} d^{-1} (r^2 = 0.998) indicate a rapid removal of both substances during wastewater treatment. These results are in agreement with those of Prasse et al.,[3] who observed removal rates in conventional wastewater treatment of 98% and >94% for acyclovir and penciclovir, respectively. Similar degradation rate constants have been determined by Joss et al.[16] for bezafibrate, fenofibric acid, N^4-acetyl-sulfamethoxazole, and gemfibrozil.

4.3.2 IDENTIFICATION OF TRANSFORMATION PRODUCTS

HPLC-UV analysis of filtered sludge samples from spiked batch experiments indicated the formation of a single TP during acyclovir degradation, whereas at least eight different TPs were observed for penciclovir. A two-step analytical procedure was applied, using i)HR-MS and HRMS/MS for potential structural elucidation and ii) NMR for final confirmation of proposed chemical structures of the TPs.

4.3.3 HR-MS AND HR-MS/MS ANALYSIS

ACV TP239. The HR-mass spectrum of ACV TP239 exhibited the same major fragment ions as the parent compound with the exception of fragment ion m/z 75 ($C_3H_7O_2^+$) which was absent and instead m/z 89 ($C_3H_5O_3^+$) was observed (SI Figure S2). This could be further fragmented (MS^3) yielding m/z 61 ($C_2H_4O_2^+$). Similar to ACV, the MS^2 spectrum of ACV TP239 showed one major product ion of m/z 152 ($C_5H_6N_5O^+$),

representing the guanine moiety and one minor product ion at m/z 164 ($C_6H_6N_5O^+$) representing methyl-guanine. The MS^2 product ions m/z 152 and m/z 164 lead to MS^3 product ions at m/z 135 ($C_5H_3N_4O^+$) and m/z 122 ($C_5H_4N_3O^+$), respectively. The close analogy between the MS spectra of both substances clearly indicates that the guanine moiety remained unchanged, whereas a modification took place at the side chain of the molecule. The mass increase of 14 Da together with results from accurate mass determinations indicated that the terminal hydroxyl group was oxidized yielding a carboxylic acid moiety.

Penciclovir TPs. Results from accurate mass measurements of penciclovir as well as its TPs are presented in Table 1, proposed fragmentation pathways are given in the SI. Similar to acyclovir, the most remarkable feature of the fragmentation patterns of all PCV TPs is the presence of the fragment ion m/z 152, indicating that the guanine moiety remained intact and that all structural changes occurred at the side chain. The results from MS^2 experiments indicated the oxidation of the side chain in two of the TPs, with one (PCV TP267) and two (PCV TP281) additional oxygen atoms being introduced into penciclovir. For PCV TP209, PCV TP233 and PCV TP237 the partial cleavage of the side chain is suggested. Further fragmentation of the side chain (MS^3) revealed different neutral losses: $[M+H-18]^+$ attributable to the cleavage of H_2O (exact mass: 18.0106 Da; PCV TP233, PCV TP237, PCV TP249B, PCV TP251), $[M+H-36]^+$ attributable to the cleavage of $2xH_2O$ (PCV 267), $[M+H-28]^+$ attributable to the loss of CO (27.9949 Da; PCV TP249A), $[M+H-44]^+$ attributable to the loss of CO2 (46.0055 Da; PCV TP209), $[M+H-88]^+$ attributable to the loss of $2xCO_2$ (PCV TP281), and $[M+H-46]^+$ attributable to the loss of formic acid (46.0055 Da; PCV TP209). Even though these losses can provide useful information for the elucidation of chemical structures of formed TPs, they were insufficient for identification. For example, the cleavage of water in positive electrospray ionization has been described for compounds containing hydroxylic moieties but also for aldehyde or carboxylic moieties.[17,18] However, in positive ESI the latter is also associated with the loss of CO_2 or HCOOH as found for PCV TP209 and PCV TP281 and aldehyde or ketone functional groups can also be indicated by the loss of CO as found for PCV TP249A. Nevertheless, based on the fragmentation patterns, the results from the accurate mass measurements and respective calculations of double bond equivalents, chemical structures could be proposed indicating the presence of carboxylic functional groups for PCV TP209, PCV TP237, PCV TP249B, and PCV TP267 as well as PCV TP281, whereas for PCV TP233, PCV TP249A, and PCV TP251 the results suggest the presence of an aldehyde moiety.

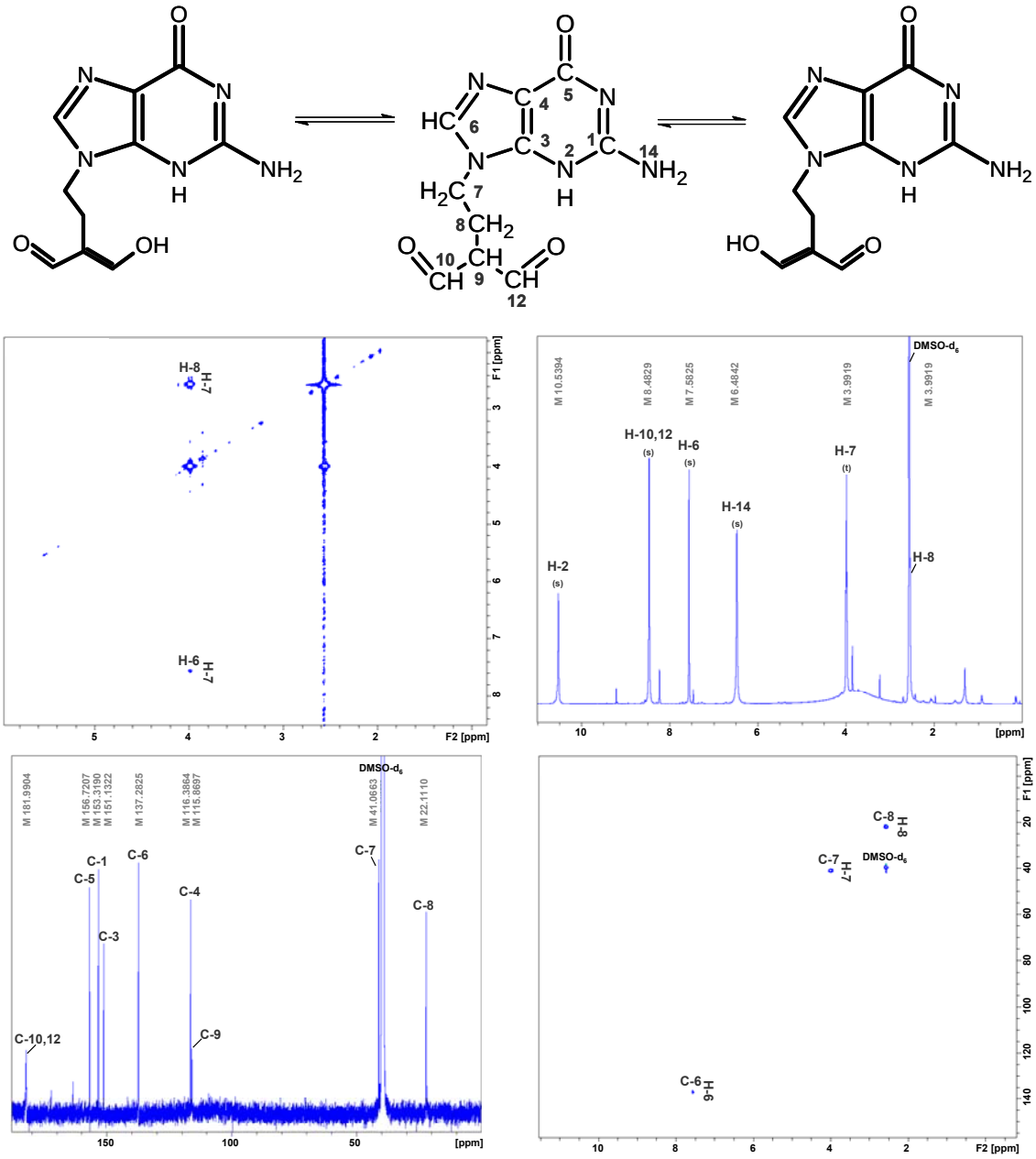

FIGURE 1. ^{1}**H-NMR (top right),** ^{1}**H,**^{1}**H-COSY (top left),** ^{1}**H,**^{13}**C-HSQC (bottom right) and** ^{13}**C-NMR spectra (bottom left) of PCV TP249A. Measurements were performed at 298.3 K in DMSO-d_6. Peak labels indicate the positions of carbon (C) and hydrogen atoms (H), numbers in light grey the respective chemical shifts.**

4.3.4 NMR Analysis

Acyclovir. ^{1}H- and ^{13}C NMR spectra of ACV TP239, the only TP of acyclovir, confirmed that the guanine moiety remains intact, whereas the primary hydroxyl group of the side chain is oxidized to the respective carboxylic acid with characteristic peaks at δ_H 12.8 ppm and δ_C 171.5 ppm in ^{1}H- and ^{13}C NMR spectra, respectively (SI Figure S8). Therefore, ACV TP239 was identified to be carboxy-acyclovir (Carboxy-ACV; 9-carboxymethoxy-methylguanine). In the following the term Carboxy-ACV is used.

Penciclovir. For seven of the identified PCV TPs a sufficient quantity could be isolated to obtain reliable NMR spectra. However, for two of these TPs (PCV TP209 and PCV TP267) amounts were insufficient to carry out ^{13}C NMR (see SI for ^{1}H NMR, ^{13}C NMR, ^{1}H,^{1}H-COSY and ^{1}H,^{13}C-HSQC spectra). The NMR spectra confirmed that the guanine moiety remains unaltered for all PCV TPs. Furthermore, with exception of PCV TP209, the chemical shifts of the first two C-atoms of the side chain (C-6 and C-7) in 13C NMR spectra were unaltered. This is supported by the coupling between H-6 and H-7 position in ^{1}H,^{1}H-COSY experiments. The presence of a carboxylic moiety was confirmed for PCV TP237, PCV TP249B, and PCV TP281, with ^{13}C-chemical shifts at δ_C 168-174 ppm. The same was true for PCV TP209 and PCV TP267 based on ^{1}H NMR, ^{1}H,^{13}C-HSQC, and ^{1}H,^{1}HCOSY spectra. For PCV TP233 and PCV TP249B, the presence of a terminal double bond was confirmed by two peaks in the 1H NMR spectra coupling to a single carbon atom in the respective ^{1}H,^{13}C-HSQC spectra. This was further supported by the ^{13}C NMR spectra with chemical shifts of the respective C-atoms being in the range typically observed for alkene-carbons (δ_C 120-140 ppm). Furthermore, ^{1}H NMR and ^{13}C NMR spectra of PCV TP233 exhibited the presence of an aldehyde functional group with characteristic chemical shifts at δ_H 9.5 ppm and δ_C 195.9 ppm, respectively. The confirmation of the chemical structure of PCV TP249A was challenging, since in the MS^2 spectra an aldehyde moiety was indicated, while in the ^{1}H NMR spectra a signal in the range δh 9.3-9.5 ppm, typical for aldehydes, was missing and instead a signal at δ_H 8.5 ppm was present. This difference is attributable to the stabilization of the 1,3-dialdehyde via keto-enol tautomerism as indicated in Figure 1. A similar feature has also been observed for malondialdehyde and malondialdehyde-acetaldehyde- protein adducts which show the same characteristic hydrogen chemical shift of about δ_H 8.5 ppm.[19,20] Furthermore, the chemical shift in the ^{13}C NMR spectrum of δ_C 182 ppm is characteristic of dialdehydes and has recently been observed for structurally related compounds such as methylglyoxal-guanine adducts.[21,22]

4.3.5 Mass Balances

Since the mass balance of the batch experiments with acyclovir was quantitative it can be concluded that Carboxy-ACV is the only TP formed in appreciable quantities (Figure 2). Even a prolongation of the test period to 29 d did not lead to a significant removal of Carboxy-ACV. For penciclovir a different behavior was observed with a mass balance that was complete until 120 h but decreased afterward to only 30% after 240 h (Figure 2). This gap can mainly be attributed to the loss of PCV TP237, with the total mass balance decreasing with decreasing concentrations of this TP (for details see below). Since the DOC decreased significantly after an initial period of 120 h, it can be assumed that the transformation of PCV TP237 either results in its mineralization or its incorporation into the microbial biomass. The latter is further supported by the fact

that guanine, which was incubated in a separate experiment, showed a complete removal (within 32 h) without any TPs being detectable (results not shown).

4.3.6 Elucidation of the Microbial Transformation Pathways

All observed transformations can be solely attributed to the presence of sludge as no transformation of parent compounds and their TPs was observed in autoclaved groundwater. The same spectrum of TPs and a similar sequence of formation was observed for experiments at low (4 µg L^{-1}) and high concentrations (20 mg L^{-1}).

Acyclovir. As mentioned previously, ACV is only undergoing oxidation of the primary hydroxyl group of the side chain of ACV, which resulted in the formation of Carboxy-ACV (Figure 3). This reaction has also been observed in patients treated with ACV.[23] In the environment, the oxidation of primary alcohols can be catalyzed by a large variety of enzymes including alcohol dehydrogenases, alcohol oxygenases, monooxygenases, and peroxidases.[24]

Penciclovir. The main transformation pathway of PCV was the oxidation of one of the primary hydroxyl groups to the respective carboxylic moiety (PCV TP267; Figure 4). PCV TP267 underwent two different transformation reactions: i) oxidation of the remaining hydroxyl group to form the respective dicarboxylic acid (PCV TP281) and/or ii) cleavage of the hydroxylated branch of the side chain yielding PCV TP237. The results from the incubation experiments with individual TPs suggest that the latter takes place via elimination of CH_2O (formaldehyde) due to the absence of PCV TP237 in incubation experiments with PCV TP281. This is further supported by results of the mass balance approach in which PCV TP237 completely disappeared at the end of the incubation period (240 h), whereas the percentage of PCV TP281 slightly increased (Figure 2). The cleavage of formaldehyde is typical for microbial degradation of compounds containing methyl or methoxyl groups[25] and has been described in detail for methyl tert-butyl ether (MTBE).[26,27] PCV TP237 is further transformed via β-oxidation resulting in the elimination of an acetyl group (PCV TP209). This reaction plays an important role in the biodegradation of fatty acids.[28]

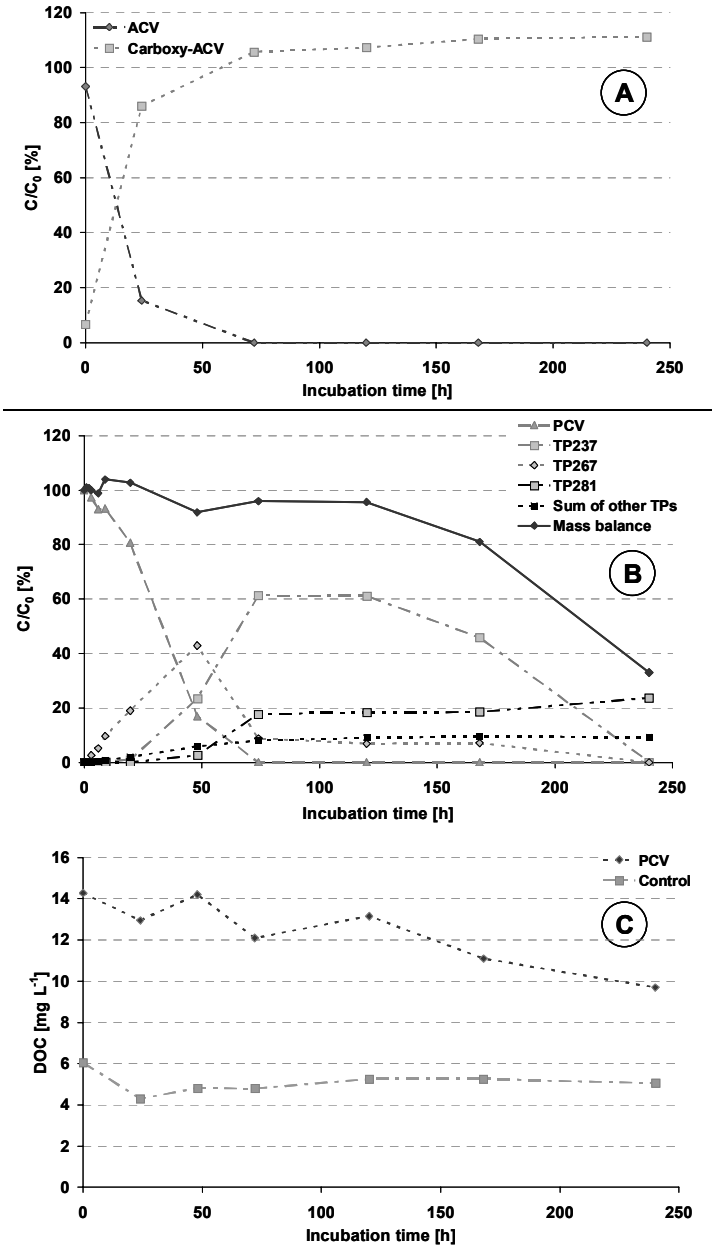

FIGURE 2. Mass balances of ACV (A) and PCV (B) in batch systems (sludge dilution 1:20) using a concentration of 20 mg L^{-1} together with results from DOC measurements obtained during PCV incubation experiments ((C); control: non-spiked sludge). Figure (B) shows the sum of PCV TP 209, TP 233, TP 252, TP 249A and TP 249 B only (sum of other TPs), as their relative individual concentrations were < 5%.

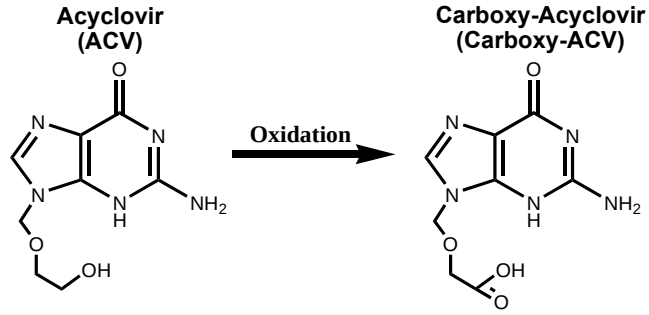

FIGURE 3. Transformation of acyclovir (ACV) in activated sludge treatment via oxidation of the primary OH-group of the side chain (Carboxy-ACV).

However, the PCV mass balance indicates that PCV TP237 also underwent transformation reactions not leading to PCV TP209 because its steep decrease after 120 h did not result in an increase of PCV TP209 (Figure 2). PCV TP209 was significantly removed when spiked individually into sludge, but no further TPs could be identified in full scans using LTQ Orbitrap Velos MS or by UV detection. As mentioned above the same is true for guanine, which was incubated in a separate experiment. Therefore, it is likely that the incorporation of TPs into the microbial biomass plays an important role in the biodegradation of penciclovir. In addition to this described major transformation pathway, two other transformations of PCV have been observed in incubation experiments: a) elimination of H_2O (dehydration) followed by the oxidation of the remaining hydroxyl group yielding an α,β-unsaturated aldehyde (PCV TP233) and b) oxidation of both primary hydroxyl groups resulting in the formation of the respective dialdehyde (PCV TP249A). The dehydration of alcohols yielding alkenes is a very significant biochemical reaction which plays an important role in the citric acid cycle.[29] Furthermore, dehydratase enzymes, such as 3-hydroxyacyl ACP dehydratase or δ-aminolevulinic acid dehydratase, also play a crucial role in fatty acid and heme synthesis.[30-32] PCV TP233 was further transformed by oxidation of the aldehyde moiety to the respective carboxylic acid (PCV TP249B) or underwent hydration yielding PCV TP251. Even though the exact position of the hydroxyl group could not be assigned in PCV TP251 as no NMR spectra were available due to the limited quantity isolated, it is likely that the hydroxyl group is located at the methyl group of the side chain which has also been observed for the hydration of acrolein yielding 3-hydroxypropanal.[33,34] However, none of the identified TPs were detected during incubation of PCV TP251, which was dissipated after 240 h. This might indicate that the hydroxyl group introduced during hydration of PCV TP233 is not located at the methyl group as proposed above but at the tertiary carbon. Incubation of PCV TP249B revealed that it can be degraded to PCV TP209; however, the exact mechanism remains unclear. Finally, incubation of PCV TP249A indicated that it is not undergoing further oxidation to the dicarboxylic acid (PCV TP281).

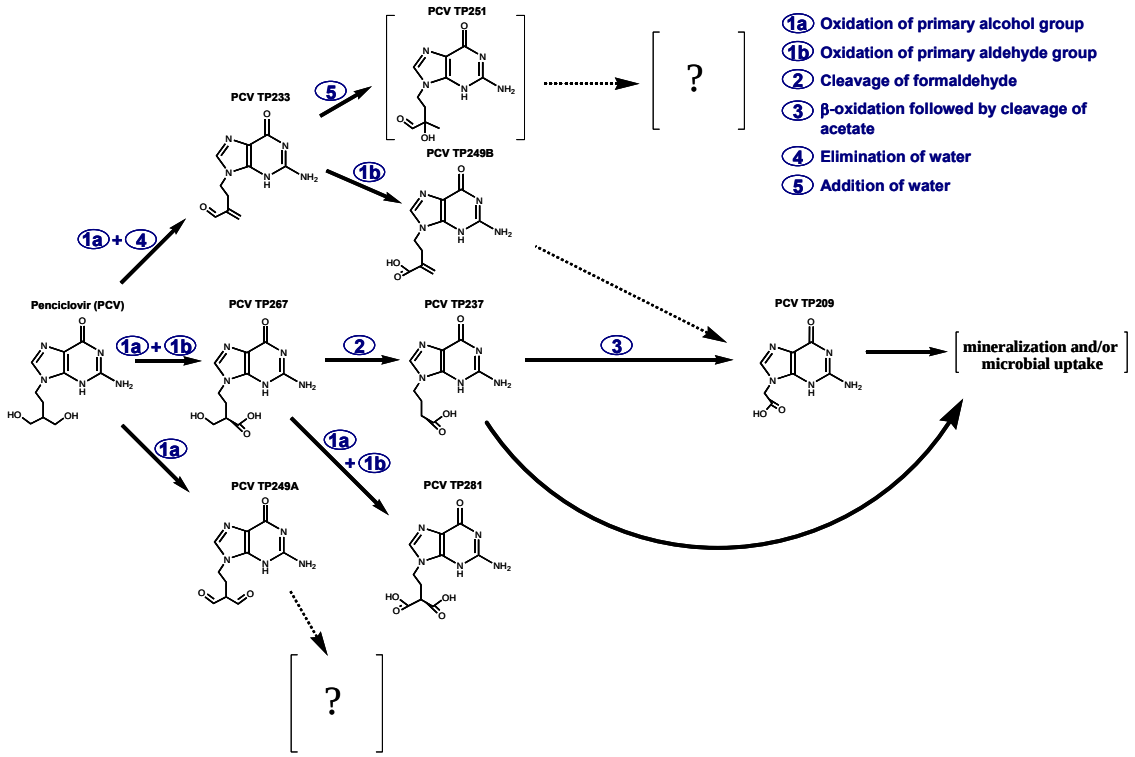

FIGURE 4. Microbial transformation pathway of penciclovir (PCV). Exact transformation pathway and sequence of TP formation was determined by incubation of individual TPs in sewage sludge. Dotted arrows indicate unknown reactions. PCV TP251 is given in parenthesis as the presented structure could not be finally confirmed due to the lack of NMR spectra.

4.3.7 Prediction of Evolving TPs via a Biotransformation Pathway Model

In recent years several models, such as CATABOL and the University of Minnesota pathway prediction system (UM-PPS), have been developed to predict biotransformation pathways of organic substances.[35,36] One potential application of these models is the screening of environmental samples for unknown substances, so-called suspect screening, which identifies transformation products of environmental micropollutants.[11,12] To predict potential TPs of acyclovir and penciclovir the UM-PPS model (http://umbbd.msi.umn.edu/predict/) was used, and results were compared with those obtained from laboratory experiments in order to assess the potential application of these models for the identification of TPs. The UM-PPS recognizes functional groups in organic compounds that are potential targets of microbial catabolic reactions and predicts transformations of these moieties based on biotransformation rules. Biotransformation rules are based on reactions found in the UM biocatalysis/biodegradation database (UMBBD) and in the scientific literature.[13] The model was allowed to predict 7 generations of aerobic and anaerobic transformations for acyclovir and penciclovir (see SI for results). In total, 40 TPs were predicted for the biotransformation of penciclovir including the three major TPs PCV TP267, PCV TP281, and PCV TP237 found in the mass balance experiments as well as PCV TP 209. For acyclovir, in total 18 TPs were predicted including Carboxy-ACV, the

only TP identified in the aerobic batch experiments. Based on these results, it can be summarized that the biotransformation model UM-PPS can be used as a starting point for the identification of the biotransformation products of ACV and PCV. However, it seems to be unable to predict all TPs formed during wastewater treatment and suggests a high number of potential TPs, which might not be relevant for environmental conditions. The latter is not surprising, considering the fact that the database (the base of this model) mainly contains biochemical reactions which have been observed for pure cultures.

4.3.8 Environmental Occurrence of Carboxy-ACV

Wastewater and Surface Water. ACV was detected in raw wastewater with concentrations up to 2.0 µg L^{-1}, whereas in treated wastewater maximum concentrations up to 0.2 µg L^{-1} were observed (Table 2). This decrease in ACV was accompanied by an increase of Carboxy-ACV for which concentrations up to 0.4 µg L^{-1} and 2.4 µg L^{-1} were observed in WWTP influents and effluents, respectively. The presence of Carboxy-ACV in WWTP influents can be explained by the human metabolism of ACV with Carboxy-ACV being the principle metabolite detected in urine of treated individuals.[37] Also the percentage of Carboxy-ACV being excreted from humans (8-14%) is consistent with results from WWTP influents (13-21%). Analysis of a large number of samples from a variety of rivers and streams (n = 53) revealed the presence of Carboxy-ACV with concentrations of up to 3.2 µg L^{-1} (median: 0.16 µg L^{-1}), clearly highlighting its widespread occurrence (SI Figure S20). Its environmental persistence indicated from activated sludge experiments was further supported by additional batch experiments with soil over a total duration of seven weeks, where no significant elimination of Carboxy-ACV was observed (Supporting Information).

TABLE 2. Concentrations of ACV and Carboxy-ACV in raw and treated wastewater (mean ± 95% confidence intervals). In addition, the sum of the molar concentrations of ACV and Carboxy-ACV in influent and effluent samples are shown.

			Concentration [ng L^{-1}]		Sum [nmol L^{-1}]
			ACV	Carboxy-ACV	
WWTP 1 (n=2)		Influent	1990	430	**10.6**
		Effluent	140	2380	**10.6**
WWTP (n=3)	**2**	Influent	1800 ± 300	247 ± 75	**9.0 ± 1.4**
		Effluent I	148 ± 11	1120 ± 180	**5.3 ± 0.8**
		Effluent II	121 ± 14	890 ± 70	**4.2 ± 0.3**

Groundwater and Drinking water. In groundwater and drinking water the concentrations of ACV were below the LOQ (see Table S2), while Carboxy-ACV was detected in all aqueous samples except for those of the anoxic groundwater (Figure 5). A comparison of the ratios of Carboxy-ACV and acesulfame concentrations in surface water and oxic groundwater confirmed the observed biological persistence of Carboxy-

ACV under aerobic conditions. In contrast, no Carboxy-ACV was detected in anoxic groundwater, indicating its degradability in the absence of oxygen. The lower ratio of Carboxy-ACV and acesulfame in finished drinking water can be explained by the mixture of treated groundwater from different oxic and anoxic wells and/or the degradation of Carboxy-ACV in drinking water treatment. Analysis of finished drinking water samples revealed the presence of Carboxy-ACV with concentrations up to 40 ng L^{-1}.

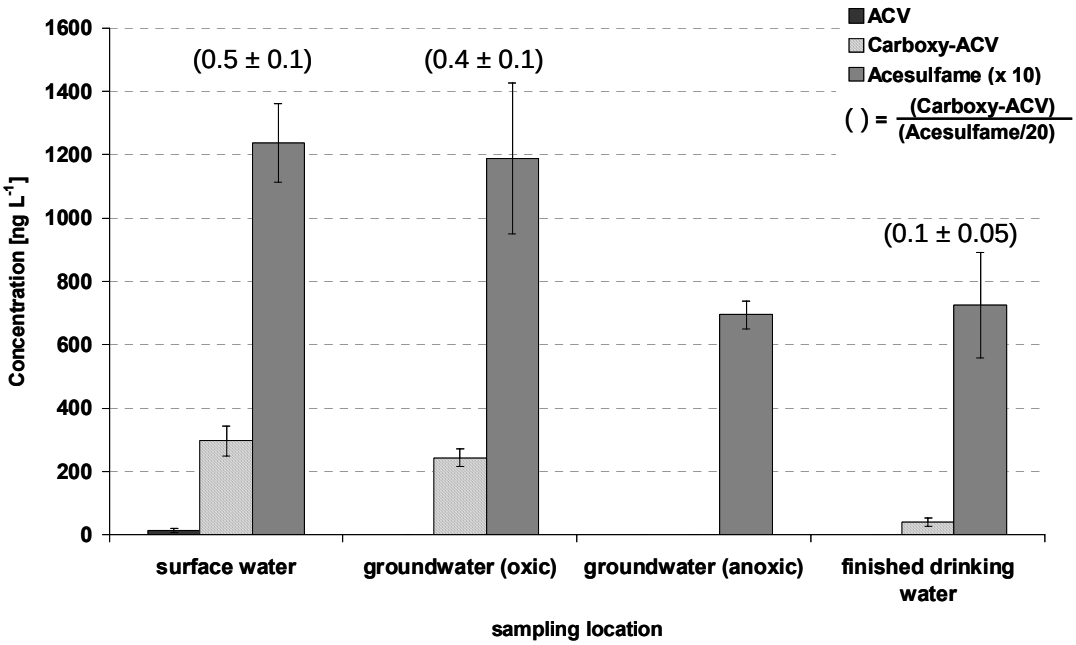

FIGURE 5. Concentrations of ACV and Carboxy-ACV along a transect ranging from surface water to a drinking water facility (n=3). Finished drinking water is obtained by mixing of treated groundwater (oxic and anoxic) from different wells. Error bars indicate 95% confidence intervals. Acesulfame was used as a stable tracer to differentiate between dilution and degradation effects. To obtain actual acesulfame concentrations the given values have to be multiplied by a factor of 10. In addition, ratios of Carboxy-ACV and acesulfame are given in parenthesis.

4.3.9 Environmental and Toxicological Relevance

The results clearly show the importance of considering TPs when investigating the fate of pharmaceuticals in the environment. The presence of Carboxy-ACV in finished drinking water might be of concern, as there is strong evidence that it is responsible for neuropsychiatric effects in patients treated with ACV.[23,38] However, concentrations in serum samples from treated individuals were in the low mg/L-range, and currently no information is available about the effects of lower doses. Several of the PCV TPs are likely to be of (eco)toxicological relevance. For instance, □,β-unsaturated aldehydes (e.g., PCV TP251) lead to enzyme inactivation, mutagenesis, protein crosslinking, and signal transduction because they are reacting with cellular nucleophiles such as glutathione (GSH), protein sidechains of cysteine, lysine and histidine and with nucleic acids.[39] Similar results have been reported for dialdehydes such as PVC TP250A. For

instance glyoxal, methyl-glyoxal, and malondialdehyde have been shown to interact with DNA.[21,22]

4.4 REFERENCES

(1) Mascolo, G.; Balest, L.; Cassano, D.; Laera, G.; Lopez, A.; Pollice, A.; Salerno, C. Biodegradability of pharmaceutical industrial wastewater and formation of recalcitrant organic compounds during aerobic biological treatment. Bioresour. Technol. 2010, 101 (8), 2585–2591.

(2) Fick, J.; Lindberg,R.H.;Tysklind, M.;Haemig, P. D.;Waldenström, J.;Wallensten, A.;Olsen, B. Antiviral oseltamivir is not removed or degraded in normal sewage water treatment: implications for development of resistance by influenza A virus. PLoS One 2007, 2 (10), e986.

(3) Prasse, C.; Schlusener, M. P.; Schulz, R.; Ternes, T. A. Antiviral drugs in wastewater and surface waters: A new pharmaceutical class of environmental relevance?. Environ. Sci. Technol. 2010, 44 (5), 1728–1735.

(4) De Clercq, E. Strategies in the design of antiviral drugs. Nat. Rev. Drug Discovery 2002, 1 (1), 13–25.

(5) Kormos, J. L.; Schulz, M.; Kohler, H. P. E.; Ternes, T. A. Biotransformation of Selected iodinated X-ray contrast media and characterization of microbial transformation pathways. Environ. Sci. Technol. 2010, 44 (13), 4998–5007.

(6) Quintana, J. B.; Weiss, S.; Reemtsma, T. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. Water Res. 2005, 39 (12), 2654–2664.

(7) Schulz, M.; Loffler, D.; Wagner, M.; Ternes, T. A. Transformation of the X-ray contrast medium lopromide in soil and biological wastewater treatment. Environ. Sci. Technol. 2008, 42 (19), 7207–7217.

(8) Celiz, M. D.; Tso, J.; Aga, D. S. Pharmaceutical metabolites in the environment: analytical challenges and ecological risks. Environ. Toxicol. Chem. 2009, 28 (12), 2473–2484.

(9) Walgren, J. L.; Mitchell, M. D.; Thompson, D. C. Role of metabolism in drug-induced idiosyncratic hepatotoxicity. Crit. Rev. Toxicol. 2005, 35 (4), 325–361.

(10) Radjenovic, J.; Sirtori, C.; Petrovic, M.; Barcelo, D.; Malato, S. Solar photocatalytic degradation of persistent pharmaceuticals at pilotscale: Kinetics and characterization of major intermediate products. Appl. Catal., B 2009, 89 (1-2), 255–264.

(11) Helbling, D. E.; Hollender, J.; Kohler, H. P. E.; Singer, H.; Fenner, K. High-throughput identification of microbial transformation products of organic micropollutants. Environ. Sci. Technol. 2010, 44 (17), 6621–6627.

(12) Kern, S.; Fenner, K.; Singer, H. P.; Schwarzenbach, R. P.; Hollender, J. Identification of transformation products of organic contaminants in natural waters by computer-aided prediction and high-resolution mass spectrometry. Environ. Sci. Technol. 2009, 43 (18), 7039–7046.

(13) Ellis, L. B. M.; Gao, J.; Fenner, K.; Wackett, L. P. The University of Minnesota pathway prediction system: predicting metabolic logic. Nucleic Acids Res. 2008, 36, W427–W432.

(14) Buerge, I. J.; Buser, H. R.; Kahle, M.; Muller, M. D.; Poiger, T. Ubiquitous occurrence of the artificial sweetener acesulfame in the aquatic environment: An ideal chemical marker of domestic wastewater in groundwater. Environ. Sci. Technol. 2009, 43 (12), 4381–4385.

(15) Scheurer, M.; Brauch, H. J.; Lange, F. T. Analysis and occurrence of seven artificial sweeteners in German waste water and surface water and in soil aquifer treatment (SAT). Anal. Bioanal. Chem. 2009, 394 (6), 1585–1594.

(16) Joss, A.; Zabczynski, S.; Gobel, A.; Hoffmann, B.; Loffler, D.; McArdell, C. S.; Ternes, T. A.; Thomsen, A.; Siegrist, H. Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. Water Res. 2006, 40 (8), 1686–1696.

(17) Tozuka, Z.; Kaneko, H.; Shiraga, T.; Mitani, Y.; Beppu, M.; Terashita, S.; Kawamura, A.; Kagayama, A. Strategy for structural elucidation of drugs and drug metabolites using (MS)(n) fragmentation in an electrospray ion trap. J. Mass Spectrom. 2003, 38 (8), 793–808.

(18) Holcapek, M.; Kolarova, L.; Nobilis, M. High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. Anal. Bioanal. Chem. 2008, 391 (1), 59–78.

(19) Kearley, M. L.; Patel, A.; Chien, J.; Tuma, D. J. Observation of a new nonfluorescent malondialdehyde-acetaldehyde-protein adduct by C-13 NMR spectroscopy. Chem. Res. Toxicol. 1999, 12 (1), 100–105.

(20) Bertz, S. H.; Dabbagh, G. Chemistry under Physiological Conditions 0.8. Nmr-Spectroscopy of Malondialdehyde. J. Org. Chem. 1990, 55 (17), 5161–5165.

(21) Pluskota-Karwatka, D.; Pawlowicz, A. J.; Bruszynska, M.; Greszkiewicz, A.; Latajka, R.; Kronberg, L. Identification of adducts formed in the reactions of malonaldehyde-glyoxal and malonaldehydemethylglyoxal with adenosine and calf thymus DNA. Chem. Biodiversity 2010, 7 (4), 959–974.

(22) Frischmann, M.; Bidmon, C.; Angerer, J.; Pischetsrieder, M. Identification of DNA adducts of methylglyoxal. Chem. Res. Toxicol. 2005, 18 (10), 1586–1592.

(23) Hellden, A.; Lycke, J.; Vander, T.; Svensson, J. O.; Odar-Cederlof, I.; Stahle, L. The aciclovir metabolite CMMG is detectable in the CSF of subjects with neuropsychiatric symptoms during acyclovir and valaciclovir treatment. J. Antimicrob. Chemother. 2006, 57 (5), 945–949.

(24) Kroutil, W.; Mang, H.; Edegger, K.; Faber, K. Biocatalytic oxidation of primary and secondary alcohols. Adv. Synth. Catal. 2004, 346 (2-3), 125–142.

(25) Orita, I.; Yurimoto, H.; Hirai, R.; Kawarabayasi, Y.; Sakai, Y.; Kato, N. The Archaeon Pyrococcus horikoshii possesses a bifunctional enzyme for formaldehyde fixation via the ribulose monophosphate pathway. J. Bacteriol. 2005, 187 (11), 3636–3642.

(26) Schmidt, T. C.; Schirmer, M.; Weiss, H.; Haderlein, S. B. Microbial degradation of methyl tert-butyl ether and tert-butyl alcohol in the subsurface. J. Contam. Hydrol. 2004, 70 (3-4), 173–203.

(27) Steffan, R. J.; McClay, K.; Vainberg, S.; Condee, C. W.; Zhang, D. L. Biodegradation of the gasoline oxygenates methyl tert-butyl ether, ethyl tert-butyl ether, and tert-amyl methyl ether by propane-oxidizing bacteria. Appl. Environ. Microbiol. 1997, 63 (11), 4216–4222.

(28) Winkler, U.; Saftel, W.; Stabenau, H. A new type of a multifunctional beta-oxidation enzyme in euglena. Plant Physiol. 2003, 131 (2), 753–762.

(29) Blank, L.; Green, J.; Guest, J. R. AcnC of Escherichia coli is a 2-methylcitrate dehydratase (PrpD) that can use citrate and isocitrate as substrates. Microbiology-(UK) 2002, 148, 133–146.

(30) Dillon, S. C.; Bateman, A. The Hotdog fold: wrapping up a superfamily of thioesterases and dehydratases. BMC Bioinf. 2004, 5, 14.

(31) White, S. W.; Zheng, J.; Zhang, Y. M.; Rock, C. O. The structural biology of type II fatty acid biosynthesis. Annu. Rev. Biochem. 2005, 74, 791–831.

(32) Korcan, S. E.; Cigerci, I. H.; Konuk, M. Screening of deltaaminolevulinic acid dehydratase from Pseudomonas strains as biosensor for lead and some other metals contamination. Environ. Monit. Assess. 2007, 134 (1-3), 263–269.

(33) Smith, A. M.; Mao, J.; Doane, R. A.; Kovacs, M. F. Metabolic fate of [C-14] Acrolein under aerobic and anaerobic aquatic conditions. J. Agric. Food Chem. 1995, 43 (9), 2497–2503.

(34) Nordone, A. J.; Matherly, R.; Bonnivier, B.; Doane, R.; Caravello, H.; Paakonen, S.; Parent, R. A. The mobility and degradation of acrolein in agricultural canals treated with magnacide(R) H herbicide. Chemosphere 1996, 32 (5), 807–814.

(35) Jaworska, J.; Dimitrov, S.; Nikolova, N.; Mekenyan, O. Probabilistic assessment of biodegradability based on metabolic pathways: Catabol system. SAR QSAR Environ. Res. 2002, 13 (2), 307–323.

(36) Hou, B. K.; Wackett, L. P.; Ellis, L. B. M. Microbial pathway prediction: A functional group approach. J. Chem. Inf. Comput. Sci. 2003, 43 (3), 1051–1057.

(37) De Miranda, P.; Good, S. S.; Krasny, H. C.; Connor, J. D.; Laskin, O. L.; Lietman, P. S. Metabolic-fate of radioactive acyclovir in humans. Am. J. Med. 1982, 73 (1A), 215–220.

(38) Tilson, H. H. Monitoring the safety of antivirals - the example of the acyclovir experience. Am. J. Med. 1988, 85 (2A), 116–122.

(39) Aldini, G.; Granata, P.; Orioli, M.; Santaniello, E.; Carini, M. Detoxification of 4-hydroxynonenal (HNE) in keratinocytes: characterization of conjugated metabolites by liquid chromatography/electrospray ionization tandem mass spectrometry. J. Mass Spectrom. 2003, 38 (11), 1160–1168.

5

OXIDATION OF THE ANTIVIRAL DRUG ACYCLOVIR AND ITS BIODEGRADATION PRODUCT CARBOXY-ACYCLOVIR WITH OZONE – KINETICS AND IDENTIFICATION OF OXIDATION PRODUCTS

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Abstract

The oxidation of the antiviral drug acyclovir (ACV) and its main biotransformation product carboxy-acyclovir (carboxy-ACV) by ozone was investigated. Both compounds have recently been detected in surface water and carboxy-ACV also in drinking water. The experiments revealed a strong pH-dependence of the oxidation of ACV and carboxy-ACV with reaction rate constants increasing by four orders of magnitude between the protonated, positively charged form ($k_{ox,PH+}$, ~ $2.5x10^2$) and the deprotonated, negatively charged form ($k_{ox,P-}$, $3.4x10^6$ M^{-1} s^{-1}). At pH 8 a single oxidation product was formed which was identified via LC-LTQ-Orbitrap MS and NMR as N-(4-carbamoyl-2-imino-5-oxoimidazolidin)formamido-N-methoxyacetic acid (COFA). Using *Vibrio fischeri* an acute bacterial toxicity was found for COFA while carboxy-ACV revealed no toxic effects. Ozonation experiments with guanine and guanosine at pH 8 led to the formation of the respective 2-imino-5-oxoimidazolidines, confirming that guanine derivatives such as carboxy-ACV are undergoing the same reactions during ozonation. Furthermore, COFA was detected in finished drinking water of a German waterworks after ozonation and subsequent activated carbon treatment.

5.1 INTRODUCTION

Since the first detection of pharmaceuticals in the environment more than three decades ago a lot of knowledge has been gained about their occurrence and fate in the urban water cycle.[1-4] Among them, antiviral drugs used for the treatment of influenza, HIV, herpes or hepatitis are a class of pharmaceuticals which have recently been detected in the environment.[5-7] For acyclovir (ACV) which is used for the treatment of herpes infections, an extensive transformation during wastewater treatment. However, this transformation leads to the formation of a stable transformation product, carboxy-acyclovir (carboxy-ACV), which was confirmed to be persistent in both wastewater treatment and in contact with sediment.[8] As a result carboxy-ACV was detected in surface water and groundwater in concentrations up to 3.2 µg L^{-1} and 0.25 µg L^{-1}, respectively. Furthermore, it was also detected in concentrations of up to 40 ng L^{-1} in finished water of a drinking water facility utilizing bank filtration followed by rapid sand filtration.[8]

Ozonation has been shown to be very efficient in eliminating a large spectrum of organic micropollutants which are not or only insufficiently removed during conventional water treatment (i.e. via sorption or (bio)degradation). This can be attributed to the reaction of ozone with chemical moieties featuring high electron densities such as double bonds.[9-12] However, several studies have shown that, similar to biological degradation, ozonation at reasonable concentrations is often not capable of fully degrading organic contaminants, thus leading to the formation of stable oxidation products (OPs)[13-17] such as aldehydes, which might be of toxicological concern.[18-20] Therefore, even though ozonation in general results in a reduced toxicity of treated compounds[21-23] and whole treated waters,[24, 25] it can also lead to the formation of oxidation products with an increased toxicity.[26-27] The formation of the carcinogen N-nitrosodimethylamine (NDMA) has been observed during ozonation of dimethylsulfamide, a degradation product of the fungicide tolylfluanide.[28] Furthermore, the formation of toxic disinfection by-products (DBPs) from natural organic matter (NOM) during ozonation and chlorination is of considerable health concern.[29] However, the knowledge about the fate of individual natural and anthropogenic organic molecules is still scarce. This is also true for modified nucleosides such as guanine analogues which are excreted in urine in considerable amounts by humans.[30, 31]

The aim of this study was to i) investigate whether ozonation is capable of removing ACV and its TP carboxy-ACV from wastewater, ii) identify evolving OPs iii) determine the bacterial toxicity of OPs formed, iv) investigate the biodegradation of OPs in a subsequent biological treatment, v) determine whether OPs can be detected in water

works with carboxy-ACV present in the raw water and vi) investigate whether similar oxidation products are formed from guanine and its derivatives such as guanosine.

5.2 MATERIALS AND METHODS

Details on used chemicals and standards, the isolation of oxidation products and the analytical method for the analysis of target analytes can be found in the SI.

5.2.1 OZONATION EXPERIMENTS

The aqueous ozone stock solution (~ 1 mM) was prepared by sparging ozone-containing oxygen through ice-bath-cooled deionized water. Ozone was generated from a O3-generator (Ozon generator 300, Fischer Technology, Germany). The concentration of the ozone stock solution was measured directly by a UV spectrometer at 258 nm using $\varepsilon_{(O3)}$ = 3000 M^{-1} cm^{-1}.

5.2.2 DETERMINATION OF PH-DEPENDENT OXIDATION KINETICS

The pH-dependent kinetics for the reaction of carboxy-ACV with ozone were performed at eight different pH-values (pH 1.7, 2.5, 3, 5, 6, 7.5, 8, 8.5). Due to the close structural relation to carboxy-ACV, degradation kinetics of ACV were only determined for three different pH-values (pH 3, 6, 8). All experiments were performed in triplicate in 50 mM phosphate buffer and at room temperature (20 ± 1°C). To exclude the influence of OH-radicals, *t*-BuOH was added as scavenger (100 mM).
For pH 1.7 - 3 reaction rate constants were determined directly using a large excess of ozone (ozone:analyte ratio > 20). The initial analyte concentration was 2 µM. Experiments were performed in 250 mL glass vessels. After the addition of ozone (end concentration ~ 40 µM), samples were taken at time intervals (every 10 – 15s) with a dispenser and were immediately quenched using indigo blue.[32] This enables to monitor the decay of ozone during the experiments. Initial and residual analyte concentrations were determined by LC-tandem MS as described in the SI.

The pseudo-first order rate constants were calculated from:

$$\ln\left(\frac{[M]}{[M_0]}\right) = -k_{obs} \cdot t \qquad \text{equation 1}$$

k_{obs} could be determined from a plot of ln([M]/[M0]) versus time with kobs being the slope of the straight line (see SI). The apparent rate constant (k_{app}) was obtained by considering the initial ozone concentration according to:

$$k_{app} = \frac{k_{obs}}{[O_3]_0}$$ equation 2

For pH-values > pH 3, rate constants were determined by competition kinetics,[33] as the reaction rates were too fast to be measurable by the method described above. Guanosine was selected as reference compound due to its close structural similarity to the target compounds. Reaction rate constants of guanosine were taken from Theravathu et al.[34] The pKa-values of the guanine moiety of guanosine, ACV and carboxy-ACV were derived from quantum-chemical calculations (pK_a1: 3.4; pK_a2: 9.6) with the protonation and deprotonation taking place at the pyrimidine ring of guanine.[35] Experiments were performed using equal amounts of the reference compound and the target compound (2 µM), but at different under-stoichiometric ozone concentrations (ranging from 0.02 to 0.2 µM).[33,36] The respective amounts of ozone were added with a glass syringe to a series of vigorously stirred beakers. After ozone was completely consumed (approx. 6h) samples were taken and analyzed via LC-tandem MS for residual concentrations of the analyte and the reference compound. For the evaluation of the data, equation 3 was used,[37, 38] where k_{O3}(R) and k_{O3}(M) are the rate constants for reference (R) and the target compound (M), respectively.

$$\ln\left(\frac{[M]}{[M(0)]}\right) = \ln\left(\frac{[R]}{[R(0)]}\right) \cdot \frac{k_{O3}(M)}{k_{O3}(R)}$$ equation 3

The rate constant k_{O3}(M) was determined from a plot of ln([M]/[M(0)]) versus ln([R]/[R(0)]) with k_{O3}(M)/k_{O3}(R) as the slope of the straight line (see SI).

To evaluate the specific rate constants for the reactions of ozone with the anionic form, the neutral form and the cationic form of carboxy-ACV the degree of dissociation has to be taken into account at each pH. Therefore, the measured apparent rate constant (k_{app}) can also be expressed as:

$$k_{app} = \alpha_+ \cdot k_{ox,PH+} + \alpha_0 \cdot k_{ox,PH} + \alpha_- \cdot k_{ox,P-}$$ equation 4

With α_+, α_0, α_- being the proportion of the cationic, neutral and anionic form at the pH selected, and $k_{ox,PH+}$, $k_{ox,PH}$ and $k_{ox,P-}$ being the rate constants towards ozone of the cationic, neutral and anionic form. The specific rate constants $k_{ox,PH}$ and $k_{ox,P-}$ were obtained by plotting α_- and α_0 against the measured rate constants in the relevant pH-range ($k_{ox,PH}$, pH 1.7 - 5; $k_{ox,P-}$, pH 6 - 8.5). The slope of the resulting straight lines represent $k_{ox,PH}$ (1.8×10^4 M^{-1} s^{-1}) and $k_{ox,P-}$ (3.4×10^6 M^{-1} s^{-1}), respectively (see SI). The good agreement with results obtained for guanosine ($k_{ox,PH}$, 1.6×10^4 M^{-1} s^{-1}; $k_{ox,P-}$, 4×10^6 M^{-1} s^{-1}) highlights its suitability as competitor for the determination of reaction

rate constants of ACV and carboxy-ACV.[34] For the specific rate constant of the protonated, positively charged form, the value for guanosine determined by Theravathu et al. was used ($k_{ox,PH+}$ ~ 2.5x10^2 M^{-1} s^{-1}).[34]

$$\alpha_+ = \frac{1}{1+10^{(pH-pKa1)}+10^{(2\cdot pH-pKa1-pKa2)}} \qquad \text{equation 5}$$

$$\alpha_0 = \frac{1}{1+10^{(pKa1-pH)}+10^{(pH-pKa2)}} \qquad \text{equation 6}$$

$$\alpha_- = \frac{1}{1+10^{(pKa1-pKa2-2\cdot pH)}+10^{(pKa2-pH)}} \qquad \text{equation 7}$$

with $\alpha_+ + \alpha_0 + \alpha_- = 1$ equation 8

5.2.3 Degradation of carboxy-ACV and Formation of OPs as a Function of the Added Quantity of Ozone

Different amounts of the ozone stock solution were added to the analyte solution (200 mL) containing carboxy-ACV in phosphate buffer (50 mM) at pH 3 or pH 8, resulting in O_3:carboxy-ACV ratios of 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1. An initial carboxy-ACV concentration of 10 µM was used to determine the formation of ozonation products dependent on the ozone doses added. To investigate the influence of OH-radicals on the formation of OPs, experiments were performed with and without the addition of *t*-BuOH (100 mM). In addition, the same experiments were performed with WWTP effluent (DOC: 12 mg L^{-1}, pH 7.7) to investigate potential matrix effects of treated wastewater.

5.2.4 Identification of oxidation products

HR-MS. Accurate MS and MS^n analyses of parent compounds and their oxidation products were performed using a Hybrid Linear Ion Trap-Orbitrap Mass Spectrometer (LTQ Orbitrap Velos, Thermo Scientific, Bremen, Germany) by direct injection using a syringe pump (flow rate: 8 µL min^{-1}) in ESI positive ion mode. To analyze samples from ozonation batch experiments directly for OPs without isolation via semi-preparative HPLC, the LTQ Orbitrap Velos was coupled to a Thermo Scientific Accela liquid chromatography system (Accela pump and autosampler). Further information on the applied setup, chromatographic conditions, and the data-dependent acquisition parameters are given in the SI. The LTQ Orbitrap Velos was used for all experiments to obtain exact masses of parent and fragment ions. External calibration was performed prior to the analysis of each batch to ensure accurate mass determinations with a resolution of 60,000. A mixture of n-butylamine, caffeine, and Ultramark 1621 (mixture of fluorinated phosphazines) was used for mass calibration. The mass accuracy was always within 0.5 ppm.

NMR. Approximately 5 mg of the isolated ozonation oxidation product formed at pH 8 (OP273) were dissolved in 0.8 mL of DMSO-d6. NMR analyses were carried out by a Bruker DRX Advance 700 instrument (Rheinstetten, Germany). ^{1}H NMR spectra were recorded at 700 MHz, ^{13}C NMR spectra at 176 MHz. Homo- and heteronuclear chemical shift correlations were determined by ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-NOESY, ^{1}H,^{13}C-HSQC, ^{1}H,^{13}C-HMBC and ^{1}H,^{15}N-HSQC. All spectra and correlation experiments were performed at 298.3 K, and chemical shifts are presented in ppm referenced to tetramethylsilane (TMS). Further details are provided in the SI.

5.2.5 Quantification of OP273 in Batch Experiments

A stock solution of OP273 at 0.1 mg mL^{-1} was prepared by dissolving one milligram of OP273 in 10 mL methanol. For the quantification of OP273 in the batch experiments, an external calibration (7 points, in phosphate buffer (50 mM, pH 8)) was used, ranging from 10 to 2,000 ng mL^{-1}. Due to the extensive Na-adduct formation $[M+Na]^+$, both $[M+H]^+$ and $[M+Na]^+$ were used for quantification (see also SI). [39]

5.2.6 Assessment of Toxicological Potential of Carboxy-ACV and OP273

The bioluminescent bacteria Vibrio fischeri were used to assess the acute bacterial toxicity of carboxy-ACV and OP273.[40, 41] To this end, different amounts (ranging from 40 – 1000 ng) of both substances were sprayed on a HPTLC plate (Silica gel 60 F 254, Merck, Darmstadt, Germany), as 6x6 mm squares, using a CAMAG (Muttenz, Switzerland) Automatic TLC Sampler 4. The TLC plate was then immersed automatically for 1 s at a speed of 3 cm s^{-1} into a solution containing Vibrio fischeri (CAMAG Immersion Device III) before monitoring the bioluminescence using a cooled CCD camera with an exposure time of 60 s (CAMAG Bioluminizer).

5.2.7 Identification of 2-imino-5-oxoimidazolidine Oxidation Products of ACV, Guanine and Guanosine

To investigate whether the respective 2-imino-5-oxoimidazolidine oxidation products are also formed during ozonation of other guanine derivatives, additional batch experiments were performed with ACV, guanine and guanosine using WWTP effluent. Analytes were spiked individually to attain an initial concentration of 10 µM of ACV, guanine and guanosine, respectively and ozone was added to attain an O_3:DOC ratio of 1:1. After the addition of ozone, samples were allowed to react for at least 2 h before 2 mL were taken and analyzed via LC-LTQ-Orbitrap MS to analyze the respective 2-imino-5-oxoimidazolidine products. Data dependent acquisition was used to obtain MS^2 and MS^3 spectra of the oxidation products thus, allowing for their identification by comparison of MS spectra with those obtained for OP273.

5.2.8 IDENTIFICATION OF OP273 IN WATER FROM A EUROPEAN WATERWORKS

A European waterworks which uses ozonation and subsequent activated carbon filtration for the production of drinking water from surface water was sampled prior and after treatment (raw water and finished drinking water). Samples (500 mL) were extracted using EnviCarb Plus cartridges (Supelco, Sigma Aldrich, Schnelldorf, Germany) at pH 8. Cartridges were conditioned using 5x2 mL methanol followed by 5x2 mL pristine groundwater (pH 8). After sample extraction, cartridges were dried under a gentle stream of nitrogen, eluted with 5x2 mL methanol + 0.2% formic acid, evaporated to 100 µL and filled up to 500 µL using 0.2% formic acid. Analysis was carried out using LC tandem MS (API 4000 QTrap, Applied Biosystems, Darmstadt, Germany) in positive MRM mode (MRM transitions for OP273: m/z 274.1 into m/z 170; m/z 141 and m/z 113). LC tandem MS conditions: total run time 27 min; ion spray 5500 V; declustering potential 46 V; collision energy (m/z 170, m/z 141, m/Z 113): 17, 29, 45 eV; collision cell exit potential 20, 8, 22 V. ESI source parameters were as follows: source temperature, 500 °C; entrance potential, 10 V; CUR, 15 psi; GS1, 20 psi; GS2, 10 psi. In addition samples were spiked with OP273 (100 µL with 1 µg mL^{-1}) to confirm the identity of detected peaks. Thee raw water was also analyzed for the presence of carboxy-ACV as described in detail in Prasse et al.[8]

5.3 RESULTS AND DISCUSSION

5.3.1 OXIDATION KINETICS OF THE REACTION OF ACV AND CARBOXY-ACV WITH OZONE

A strong pH-dependence of the reactivity of ACV and carboxy-ACV with ozone was observed, attributable to the pH-dependence of the amine moieties present in the molecules (Fig. 1). Due to the addition of *t*-BuOH as scavenger the reaction with OH-radicals can be excluded. Protonated amines (present at low pH-values) have been shown to react very slowly with ozone (second order rate constants in general ranging from 10^2 to 10^3 M^{-1} s^{-1}). The deprotonated amine moiety exhibited second order rate constants in the range of 10^5-10^6 M^{-1} s^{-1}, due to the increased electron density.[34, 42, 43] Determination of the specific rate constants $k_{ox,PH}$ and $k_{ox,P-}$ for carboxy-ACV revealed an increase of two orders of magnitude from the neutral ($k_{ox,PH}$ $1.8x10^4$ M^{-1} s^{-1}) to the deprotonated form ($k_{ox,P-}$ $3.4x10^6$ M^{-1} s^{-1}), respectively indicating the rapid reaction of carboxy-ACV and ACV with ozone under typical treatment conditions for both drinking water and treated wastewater (pH 7-8).

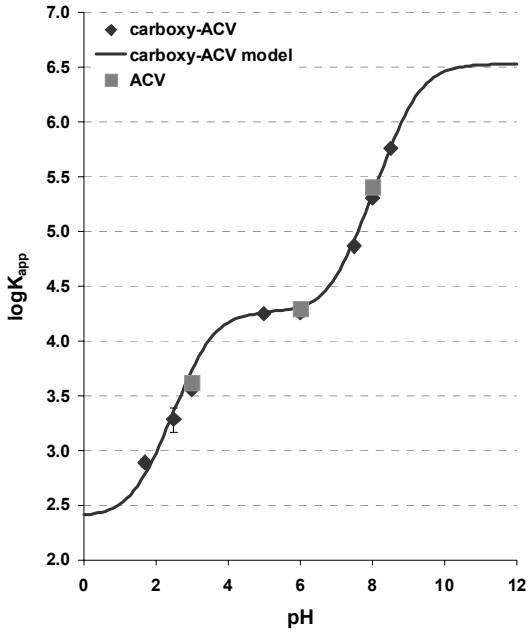

FIGURE 1. pH-dependent rate constants ($logk_{app}$) of carboxy-ACV and ACV for the reaction with ozone. The solid line are the calculated rate constants based on rate constants of the cationic ($k_{ox,PH+}$), the neutral ($k_{ox,PH}$) and the anionic form ($k_{ox,P-}$).

5.3.2 IDENTIFICATION OF OP273 FORMED DURING OZONATION AT PH 8

HR-MS. In total, 8 fragments were observed in MS^n spectra of OP273 (see SI for details). The molecular ion peak at m/z 274 was fragmented (MS^2) into m/z 198 and 134. Cleavage of $C_2H_4O_3$ from m/z 274 leading to fragment m/z 198 indicates that the side chain of the molecule remained intact as a similar behavior has also been observed for carboxy-ACV.[8] Fragment m/z 198 could be further fragmented (MS^3) by cleavage of CO yielding m/z 170, indicating the presence of an aldehyde moiety in the molecule. Furthermore, the presence of an primary amine moiety is indicated by the cleavage of NH_3 from m/z 170 yielding m/z 153 (MS^4).

NMR. In accordance with results from HR-MS, the NMR spectra of OP273 revealed that the side chain attached to the guanine moiety remained unchanged as the broad shift at 12-14 ppm, characteristic for the presence of a carboxylic moiety, and chemical shifts of two CH_2-groups (C-10 and C-11) were still present (Fig. 2). However, in contrast to carboxy-ACV chemical shifts of protons attached to C-10 at 4.9 ppm and 5.2 ppm in COFA differ quite substantially, indicating a hindered rotation.[44] The same is true for protons at 7.2 and 7.3 ppm in the ^{1}H-NMR spectrum of OP273 which were assigned to the formamido N-7 (see also ^{1}H,^{15}N-HSQC spectrum; Fig. 2). Jean et al. showed that the energy barrier for the rotation of the protons in acetamides is 15 kcal mol^{-1}.[45] Furthermore, a similar behavior has been observed by Ye et al. for 5-carboxamido-5-formamido-2-iminohydantoin.[46] An additional coupling in

$^{1}H,^{15}N$-HSQC spectrum of OP273 was observed which was assigned to the amine group (N-13). The absence of the coupling of the protons at N-1 and N-3 in the $^{1}H,^{15}N$-HSQC spectrum can be attributed to an imine-enamine tautomerism and the resulting relocation of the double bond between N-1, C-2, N-3 and N-13. Finally, proton chemical shift at 8.0 ppm could be assigned to the aldehyde moiety (C-9) of the molecule. Even though aldehyde chemical shifts are typically observed in the range of 9.2 - 9.5 ppm, the difference can be attributed to the presence of the amine moiety in α-position.[47] Therefore, results from NMR allowed for the unambiguous identification of OP273 as N-(4-carbamoyl-2-imino-5-oxoimidazolidin)formamido-N-methoxyacetic acid (COFA; alternative name: 4-((N-formamido)-N-methoxy acetyl)-2-imino-5-oxo-imidazolidin-4-carboxylamide). In the following the term COFA is used.

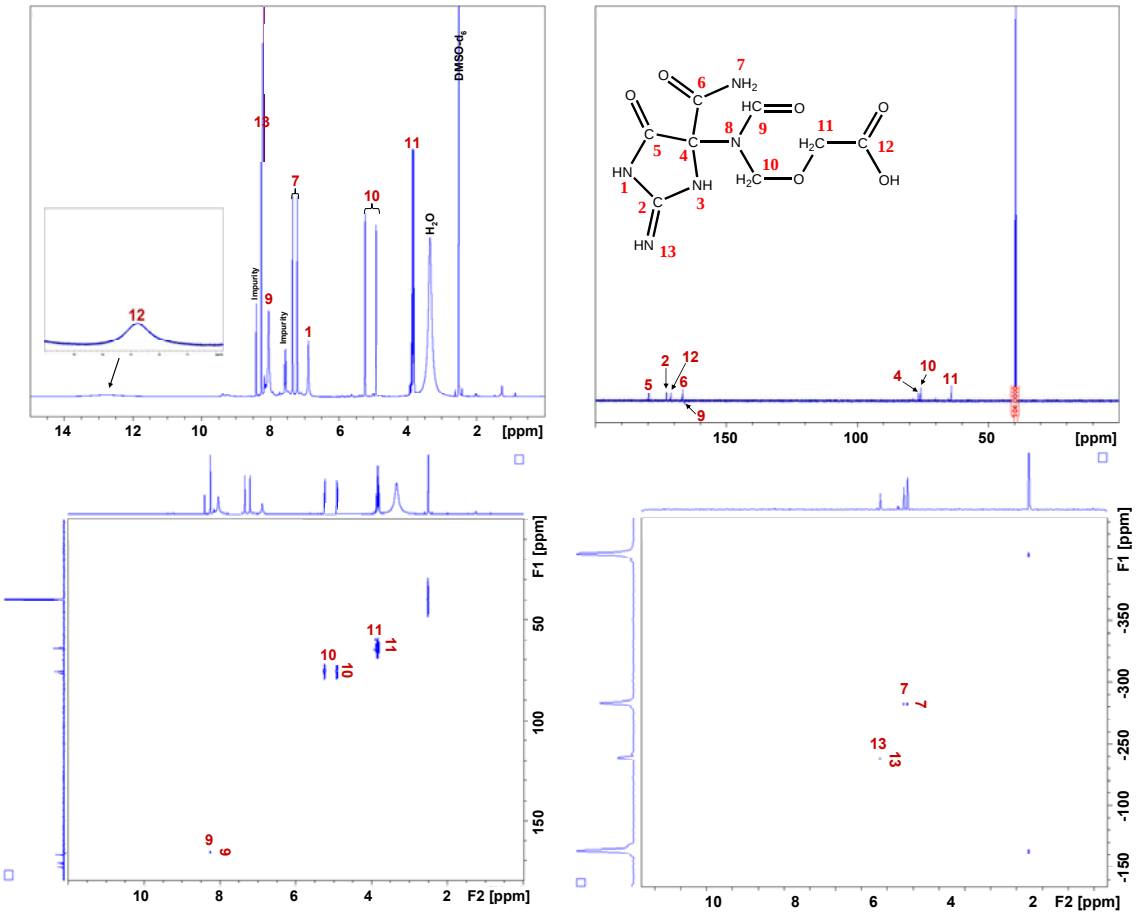

Figure 2. ^{1}H-NMR (top, left), ^{13}C-NMR (top, right), $^{1}H,^{13}C$-HSQC (bottom, left) and $^{1}H,^{15}N$-HSQC (bottom, right) spectra of COFA. $^{1}H,^{1}H$-COSY, $^{1}H,^{1}H$-NOESY and $^{1}H,^{13}C$-HMBC spectra are given in the SI. All experiments were performed at 298 K.

5.3.3 PROPOSED REACTION MECHANISM LEADING TO THE FORMATION OF COFA

The proposed reaction mechanism leading to the formation of COFA is shown in Fig. 3. The structures of postulated intermediate products are based on already known ozone reactions reported in literature and can be suggested in the following sequence. Oxidative attack by ozone of the double bond at the fusion of the pyrimidine and the imidazole ring (C-4/C-5) should result in the formation an ozonide. The ozonide reacts further by the release of singlet oxygen ($^1O_2$), thus forming an epoxide-intermediate. This is supported by results from Enami et al.[48] who described comparable reactions for the ozonolysis of uric acid and Ye et al.[46] who observed the respective 2-imino-5-oxoimidazolidine product for the reaction of guanine with the epoxidizing agent dimethyldioxirane. Furthermore, the release of $^1O_2$ has already been observed for the reaction of guanosine with O_3.[49, 50] The epoxide-intermediate can further undergo a 1,2-acyl shift, leading to the formation of a quarternary carbon, which is followed by the hydrolytic opening of the imidazole ring,[46, 51] finally leading to the formation of the COFA.

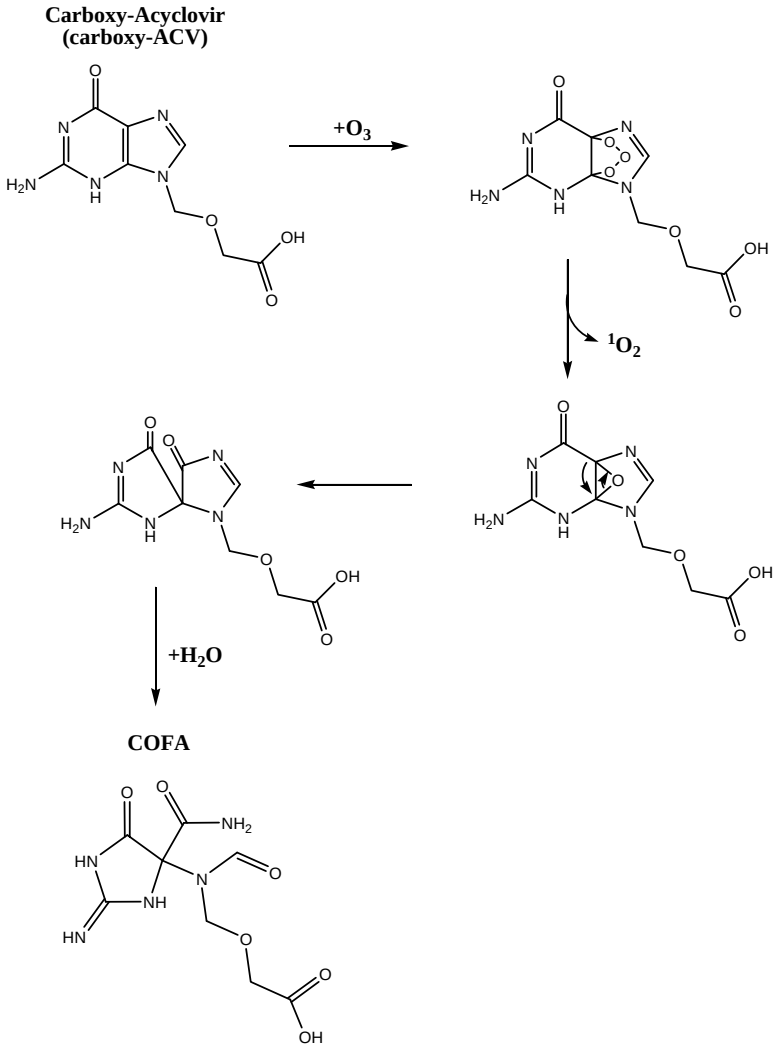

FIGURE 3. Proposed reaction mechanism of carboxy-ACV with ozone at pH 8 leading to the formation of COFA.

5.3.4 FORMATION OF COFA WITH VARIOUS OZONE DOSES

The transformation of carboxy-ACV to COFA at different O_3:carboxy-ACV ratios with and without the addition of *t*-BuOH is shown in Fig. 4 (top). An increased formation of COFA is observed with an elevated O_3:carboxy-ACV ratio until a complete conversion of carboxy-ACV to COFA is obtained independent of the addition of the radical scavenger *t*-BuOH. Furthermore, it is indicated that COFA is resistant to further oxidative transformation via ozone, even at higher ozone doses. However, in WWTP effluent (Fig 4, bottom) a decrease of COFA was observed for higher ozone doses. This might be caused by i) the formation of reactive species formed by the reaction of ozone with other constituents present in the WWTP effluent which react further with COFA and/or ii) the effluent organic matter (EfOM)-promoted decomposition of O_3 resulting in the increased formation of OH-radicals.[52, 53]

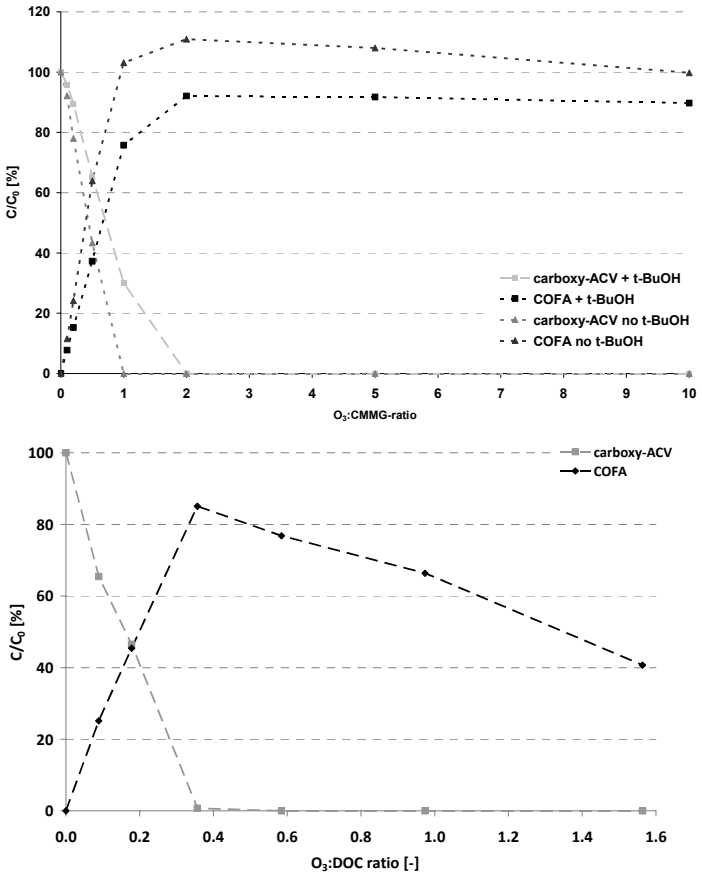

FIGURE 4. Formation of COFA during ozonation of carboxy-ACV in phosphate buffer (50 mM, pH 8) with and without the addition of t-BuOH as radical scavenger (top) and in WWTP effluent (DOC 10 mg L^{-1}, pH 7.7) (bottom). Initial carboxy-ACV concentration was 10 µmol L^{-1} in all experiments.

5.3.5 Bacterial Toxicity of Carboxy-ACV and COFA

The toxicity screening of carboxy-ACV using V. fischeri exhibited that no bacterial toxicity was observed for all quantities (40 - 1000 ng) of carboxy-ACV sprayed on the HPTLC plate (Fig. 5). However, with COFA an inhibition of the bacteria was observed, indicating that ozonation of carboxy-ACV results in the formation of a bacterially toxic oxidation product (COFA). There is a reasonable likelihood that the aldehyde moiety of COFA is responsible for the toxic effects observed e.g. due to Schiff base formation.[54,55] However, additional experiments are necessary to elucidate the underlying mechanisms leading to the observed acute bacterial toxicity of COFA.

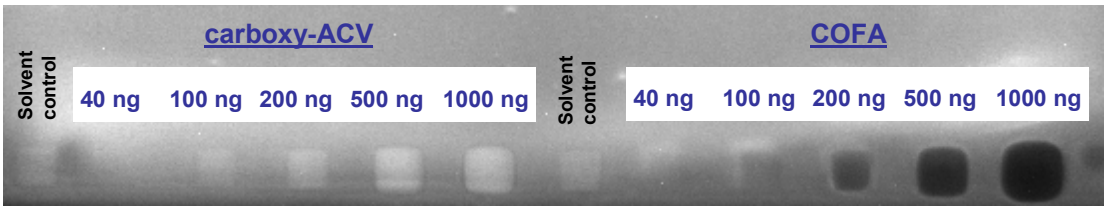

FIGURE 5. Inhibition of *V. fischeri* on HPTLC plates sprayed with different amounts of carboxy-ACV (left) and COFA (right). Different volumes (2 µL, 5 µL, 10 µL, 25 µL and 50 µL) of a 20 µg mL^{-1} stock solution of both analytes were sprayed on the HPTLC plate using an automatic TLC sampler (CAMAG, ATS4). For detection of bioluminescence a cooled CCD camera (CAMAG BioLuminizer) with an exposure time of 60 s was used.

5.3.6 Degradation of COFA in Subsequent Biological Treatment

Batch experiments were performed with activated sludge to investigate whether the formed COFA can be further biodegraded in a subsequent biological treatment. It was suggested by several authors, that ozonation is capable of oxidizing and breaking up recalcitrant molecules and that the resulting oxidation products can then be further transformed in a subsequent biological treatment.[56-59] In order to evaluate this assumption, activated sludge was taken from a conventional municipal WWTP and COFA was added to attain an initial concentration of 200 µg L^{-1}. Detailed information on the experimental setup used can be found in Prasse et al.[8] However, for COFA no significant biodegradation was observed within 48 h (a maximal hydraulic retention time) indicating the persistence of COFA under conventional biological treatment conditions (Fig. 6). Even an incubation of up to 14 days only resulted in an elimination of COFA of approx. 40 % indicating that a biological degradation of COFA is unlikely in biological wastewater treatment and presumably in sand filtration as well. Therefore, COFA is one of the first examples indicating that ozonation might also result in the formation of toxic OPs are recalcitrant in subsequent biological treatment processes. However, in order to generalize these results, further studies with a high number of ozonation products are crucial.

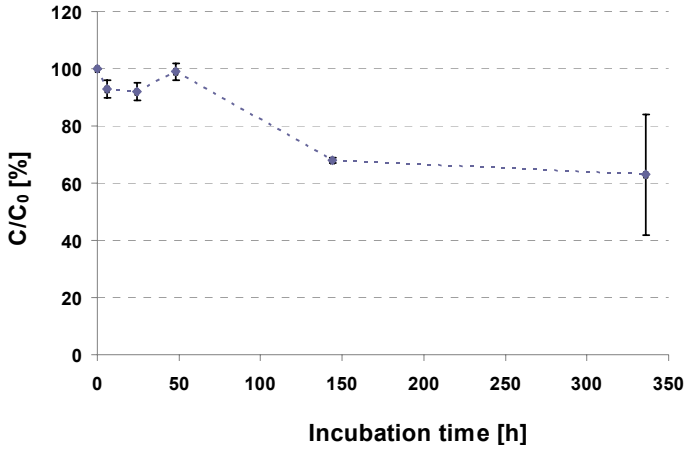

FIGURE 6. Degradation of COFA in sewage sludge taken from the aerobic unit of a conventional municipal wastewater treatment plant (n=2). Total test duration was 336 h (14 days). Sludge was diluted to obtain an initial sludge concentration of 0.25 g_{TSS} L^{-1}, initial concentration of COFA was 200 µg L^{-1}. The vessels were permanently aerated and stirred to maintain aerobic conditions. In addition, a small amount of CO_2 was added to the air to maintain a stable pH (pH 7).

5.3.7 OZONATION OF ACV, GUANINE AND GUANOSINE IN WWTP EFFLUENT

To evaluate whether the proposed reaction with ozone is also relevant for other guanine derivatives, additional ozonation experiments were performed with ACV, guanine and guanosine. For all three compounds the corresponding 2-imino-5-oxoimidazolidine analogues were formed (Fig. 7). The chemical structure of the 2-imino-5-oxoimidazolidine formed from guanine was further confirmed by comparison of the MS spectra with those obtained by Ye et al. for the reaction of guanine with the epoxidizing agent DMDO.[46] As the 2-imino-5-oxoimidazolidine moiety is most likely responsible for the observed acute bacterial toxicity of COFA, similar results can be expected for the oxidation products formed during ozonation of ACV, guanine and guanosine.

5.3.8 IMPLICATIONS OF THE FATE OF CARBOXY-ACV DURING OZONATION IN WASTEWATER TREATMENT AND IN DRINKING WATER PURIFICATION

As indicated in Fig. 4 an ozone:DOC ratio of approx. 0.4 is necessary to attain a complete elimination of carboxy-ACV indicating a complete degradation under typical treatment conditions. Similar results are expected with regard to drinking water treatment via ozone. Even though the DOC is in general significantly lower in the feed water used by waterworks, the obtained results suggest that COFA is also formed under these conditions due to a probably insignificant effect of DOC as an OH-scavenger since t-BuOH did not significantly influence the ozonation results. In addition, comparison of results from buffered ultrapure water and WWTP effluent indicates a high stability of COFA even at very high ozone doses in water with a low DOC content.

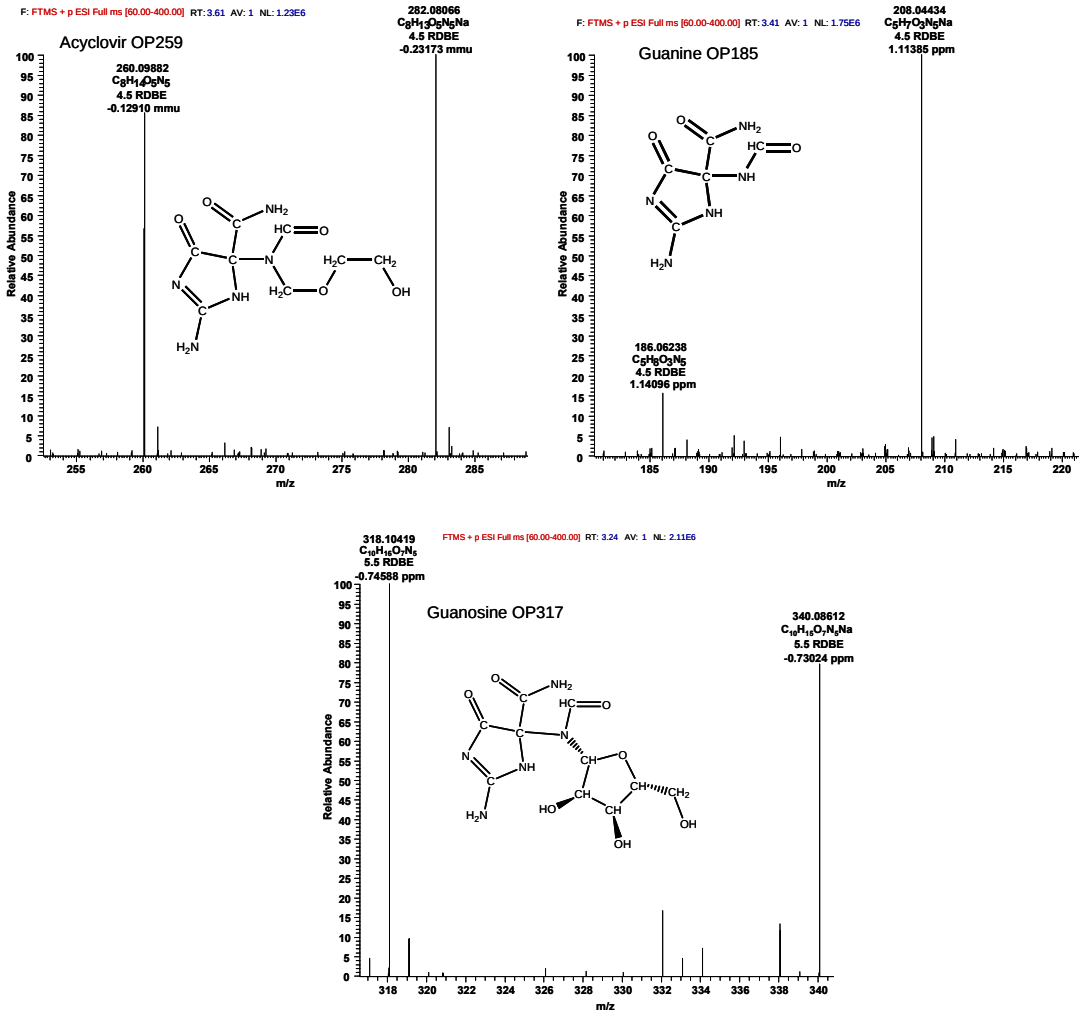

FIGURE 7. Formation of 2-imino-5-oxoimidazolidine oxidation products during ozonation of ACV (top left) and guanine (top right) and guanosine (bottom) in WWTP effluent (DOC 12 mg L^{-1}, pH 7.7). Similar to OP273, the extensive formation of the $[M+Na]^+$-adducts was observed. Initial concentration of ACV, guanine and guanosine was 10 µmol L^{-1}, respectively. Ozone was added to attain a O_3:DOC ratio of 1:1. MS^2 spectra of the ozonation products are given in the SI.

5.3.9 Formation of COFA during Ozonation in a Full-scale Plant

Feeding water and the finished drinking water of a German waterworks (using ozonation and subsequent activated carbon filtration) were analyzed for carboxy-ACV and COFA. Carboxy-ACV was found in the feeding water at a concentration of 82 ng L^{-1}. As shown in Fig. 8 the ozonation product COFA was present in the final drinking water after ozonation and granular activated carbon (GAC) filtration. As the analytical method for COFA was not validated, an exact quantification was not feasible. However, the results indicate that COFA is formed during ozonation in this waterworks in appreciable quantities. Furthermore, the presence of COFA in finished drinking water indicates that the results of the laboratory experiments can be transferred to real waterworks conditions and that COFA is even prone to pass GAC filters.

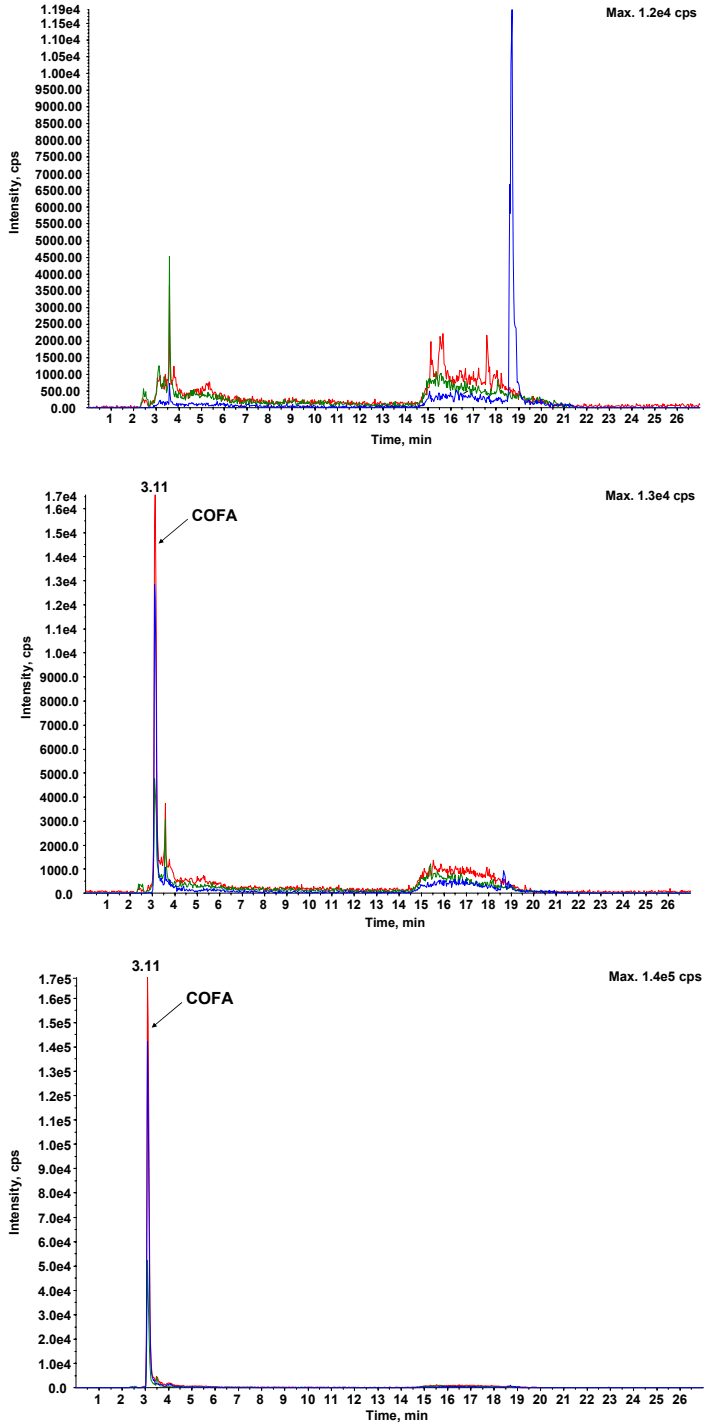

FIGURE 8. LC tandem MS chromatograms of COFA (retention time 3.11 min) in raw water (top), finished drinking water after ozonation and activated carbon treatment (middle) and in finished drinking water spiked with 100 ng of COFA (bottom). MRM transitions: 274.1 → 170 (blue); 274.1 → 141 (red); 274.1 → 113 (green).

5.4 REFERENCES

(1) Daughton, C. G.; Ternes, T. A., Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environ. Health Perspect. 1999, 107, 907-938.

(2) Benotti, M. J.; Trenholm, R. A.; Vanderford, B. J.; Holady, J. C.; Stanford, B. D.; Snyder, S. A., Pharmaceuticals and Endocrine Disrupting Compounds in US Drinking Water. Environ. Sci. Technol. 2009, 43 (3), 597-603.

(3) Kummerer, K., Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - a review. Chemosphere 2001, 45 (6-7), 957-969.

(4) Richardson, S. D.; Ternes, T. A., Water Analysis: Emerging Contaminants and Current Issues. Anal. Chem. 2011, 83 (12), 4614-4648.

(5) Ghosh, G. C.; Nakada, N.; Yamashita, N.; Tanaka, H., Oseltamivir Carboxylate, the Active Metabolite of Oseltamivir Phosphate (Tamiflu), Detected in Sewage Discharge and River Water in Japan. Environ. Health Perspect. 2010, 118 (1), 103-107.

(6) Prasse, C.; Schluesener, M. P.; Schulz, R.; Ternes, T. A., Antiviral Drugs in Wastewater and Surface Waters: A New Pharmaceutical Class of Environmental Relevance? Environ. Sci. Technol. 2010, 44 (5), 1728-1735.

(7) Soderstrom, H.; Jarhult, J. D.; Olsen, B.; Lindberg, R. H.; Tanaka, H.; Fick, J., Detection of the Antiviral Drug Oseltamivir in Aquatic Environments. PLoS One 2009, 4 (6).

(8) Prasse, C.; Wagner, M.; Schulz, R.; Ternes, T. A., Biotransformation of the Antiviral Drugs Acyclovir and Penciclovir in Activated Sludge Treatment. Environ. Sci. Technol. 2011, 45 (7), 2761-2769.

(9) Gerrity, D.; Snyder, S., Review of Ozone for Water Reuse Applications: Toxicity, Regulations, and Trace Organic Contaminant Oxidation. Ozone-Science & Engineering 2011, 33 (4), 253-266.

(10) Hollender, J.; Zimmermann, S. G.; Koepke, S.; Krauss, M.; McArdell, C. S.; Ort, C.; Singer, H.; von Gunten, U.; Siegrist, H., Elimination of Organic Micropollutants in a Municipal Wastewater Treatment Plant Upgraded with a Full-Scale Post-Ozonation Followed by Sand Filtration. Environ. Sci. Technol. 2009, 43 (20), 7862-7869.

(11) Ternes, T. A.; Stuber, J.; Herrmann, N.; McDowell, D.; Ried, A.; Kampmann, M.; Teiser, B., Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? Water Res. 2003, 37 (8), 1976-1982.

(12) von Gunten, U., Ozonation of drinking water: Part I. Oxidation kinetics and product formation. Water Res. 2003, 37 (7), 1443-1467.

(13) Benner, J.; Ternes, T. A., Ozonation of Metoprolol: Elucidation of Oxidation Pathways and Major Oxidation Products. Environ. Sci. Technol. 2009, 43 (14), 5472-5480.

(14) Benner, J.; Ternes, T. A., Ozonation of Propranolol: Formation of Oxidation Products. Environ. Sci. Technol. 2009, 43 (13), 5086-5093.

(15) Dodd, M. C.; Rentsch, D.; Singer, H. P.; Kohler, H.-P. E.; von Gunten, U., Transformation of beta-Lactam Antibacterial Agents during Aqueous Ozonation: Reaction Pathways and Quantitative Bioassay of Biologically-Active Oxidation Products. Environ. Sci. Technol. 2010, 44 (15), 5940-5948.

(16) McDowell, D. C.; Huber, M. M.; Wagner, M.; Von Gunten, U.; Ternes, T. A., Ozonation of carbamazepine in drinking water: Identification and kinetic study of major oxidation products. Environ. Sci. Technol. 2005, 39 (20), 8014-8022.

(17) Scheurer, M.; Godejohann, M.; Wick, A.; Happel, O.; Ternes, T. A.; Brauch, H.-J.; Ruck, W. K. L.; Lange, F. T., Structural elucidation of main ozonation products of the artificial sweeteners cyclamate and acesulfame. Environmental Science and Pollution Research 2011, DOI 10.1007/s11356-011-0618-x.

(18) Can, Z. S.; Gurol, M., Formaldehyde formation during ozonation of drinking. Ozone-Science & Engineering 2003, 25 (1), 41-51.

(19) Nawrocki, J.; Swietlik, J.; Raczyk-Stanislawiak, U.; Dabrowska, A.; Bilozor, S.; Ilecki, W., Influence of ozonation conditions on aldehyde and carboxylic acid formation. Ozone-Science & Engineering 2003, 25 (1), 53-62.

(20) Wert, E. C.; Rosario-Ortiz, F. L.; Drury, D. D.; Snyder, S. A., Formation of oxidation byproducts from ozonation of wastewater. Water Res. 2007, 41 (7), 1481-1490.

(21) Dodd, M. C.; Kohler, H. P. E.; Von Gunten, U., Oxidation of Antibacterial Compounds by Ozone and Hydroxyl Radical: Elimination of Biological Activity during Aqueous Ozonation Processes. Environ. Sci. Technol. 2009, 43 (7), 2498-2504.

(22) Gomez-Ramos, M. d. M.; Mezcua, M.; Aguera, A.; Fernandez-Alba, A. R.; Gonzalo, S.; Rodriguez, A.; Rosal, R., Chemical and toxicological evolution of the antibiotic sulfamethoxazole under ozone treatment in water solution. Journal of hazardous materials 2011, 192 (1), 18-25.

(23) Li, K.; Yediler, A.; Yang, M.; Schulte-Hostede, S.; Wong, M. H., Ozonation of oxytetracycline and toxicological assessment of its oxidation by-products. Chemosphere 2008, 72 (3), 473-478.

(24) Bundschuh, M.; Pierstorf, R.; Schreiber, W. H.; Schulz, R., Positive Effects of Wastewater Ozonation Displayed by in Situ Bioassays in the Receiving Stream. Environ. Sci. Technol. 2011, 45 (8), 3774-3780.

(25) Stalter, D.; Magdeburg, A.; Wagner, M.; Oehlmann, J., Ozonation and activated carbon treatment of sewage effluents: Removal of endocrine activity and cytotoxicity. Water Res. 2011, 45 (3), 1015-1024.

(26) Von Gunten, U.; Salhi, E.; Schmidt, C. K.; Arnold, W. A., Kinetics and Mechanisms of N-Nitrosodimethylamine Formation upon Ozonation of N,N-Dimethylsulfamide-Containing Waters: Bromide Catalysis. Environ. Sci. Technol. 2010, 44 (15), 5762-5768.

(27) Yang, L.; Chen, Z.; Shen, J.; Xu, Z.; Liang, H.; Tian, J.; Ben, Y.; Zhai, X.; Shi, W.; Li, G., Reinvestigation of the Nitrosamine-Formation Mechanism during Ozonation. Environ. Sci. Technol. 2009, 43 (14), 5481-5487.

(28) Schmidt, C. K.; Brauch, H.-J., N,N-dimethosulfamide as precursor for N-nitrosodimethylamine (NDMA) formation upon ozonation and its fate during drinking water treatment. Environ. Sci. Technol. 2008, 42 (17), 6340-6346.

(29) Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; DeMarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: A review and roadmap for research. Mutation Research-Reviews in Mutation Research 2007, 636 (1-3), 178-242.

(30) Cho, S.-H.; Choi, M. H.; Lee, W.-Y.; Chung, B. C., Evaluation of urinary nucleosides in breast cancer patients before and after tumor removal. Clinical Biochemistry 2009, 42 (6), 540-543.

(31) Ito, T.; van Kuilenburg, A. B. P.; Bootsma, A. H.; Haasnoot, A. J.; van Cruchten, A.; Wada, Y.; van Gennip, A. H., Rapid screening of high-risk patients for disorders of purine and pyrimidine metabolism using HPLC-electrospray tandem mass spectrometry of liquid urine or urine-soaked filter paper strips. Clinical Chemistry 2000, 46 (4), 445-452.

(32) Bader, H.; Hoigne, J., Determination of Ozone in Water by the Indigo Method. Water Res. 1981, 15 (4), 449-456.

(33) Huber, M. M.; Canonica, S.; Park, G. Y.; Von Gunten, U., Oxidation of pharmaceuticals during ozonation and advanced oxidation processes. Environ. Sci. Technol. 2003, 37 (5), 1016-1024.

(34) Theruvathu, J. A.; Flyunt, R.; Aravindakumar, C. T.; von Sonntag, C., Rate constants of ozone reactions with DNA, its constituents and related compounds. J. Chem. Soc.-Perkin Trans. 2 2001, (3), 269-274.

(35) Verdolino, V.; Cammi, R.; Munk, B. H.; Schlegel, H. B., Calculation of pK(a) Values of Nucleobases and the Guanine Oxidation Products Guanidinohydantoin and Spiroiminodihydantoin using Density Functional Theory and a Polarizable Continuum Model. Journal of Physical Chemistry B 2008, 112 (51), 16860-16873.

(36) Benner, J.; Salhi, E.; Ternes, T.; von Gunten, U., Ozonation of reverse osmosis concentrate: Kinetics and efficiency of beta blocker oxidation. Water Res. 2008, 42 (12), 3003-3012.

(37) Benitez, F. J.; Beltran-Heredia, J.; Acero, J. L.; Rubio, F. J., Rate constants for the reactions of ozone with chlorophenols in aqueous solutions. Journal of Hazardous Materials 2000, 79 (3), 271-285.

(38) Munoz, F.; von Sonntag, C., Determination of fast ozone reactions in aqueous solution by competition kinetics. J. Chem. Soc.-Perkin Trans. 2 2000, 4, 661-664.

(39) Li, X. F.; Ma, M. S.; Scherban, K.; Tam, Y. K., Liquid chromatography-electrospray mass spectrometric studies of ginkgolides and bilobalide using simultaneous monitoring of proton, ammonium and sodium adducts. Analyst 2002, 127 (5), 641-646.

(40) Eberz, G.; Rast, H. G.; Burger, K.; Kreiss, W.; Weisemann, C., Bioactivity screening by chromatography-bioluminescence coupling. Chromatographia 1996, 43 (1-2), 5-9.

(41) Schulz, W.; Seitz, W.; Weiss, S. C.; Weber, W. H.; Bohm, M.; Flottmann, D., Use of Vibrio fischeri for Screening for Bioactivity in Water Analysis. JPC-J. Planar Chromatogr.-Mod. TLC 2008, 21 (6), 427-430.

(42) Hoigne, J.; Bader, H., Rate Constants of Reactions of Ozone with Organic and Inorganic-Compounds in Water .2. Dissociating Organic-Compounds. Water Res. 1983, 17 (2), 185-194.

(43) Munoz, F.; von Sonntag, C., The reactions of ozone with tertiary amines including the complexing agents nitrilotriacetic acid (NTA) and ethylenediaminetetraacetic acid (EDTA) in aqueous solution. J. Chem. Soc.-Perkin Trans. 2 2000, (10), 2029-2033.

(44) Mancilla, T.; Zamudio-Rivera, L. S.; Carrillo, L.; Beltran, H. I.; Farfan, N., Synthesis and characterization of new 2-(alkylamino)acetamides. Arkivoc 2003, 37-47.

(45) Jean, Y.; Demachy, I.; Lledos, A.; Maseras, F., Electronic against steric effects in distorted amides. Journal of Molecular Structure-Theochem 2003, 632, 131-144.

(46) Ye, W. J.; Sangaiah, R.; Degen, D. E.; Gold, A.; Jayaraj, K.; Koshlap, K. M.; Boysen, G.; Williams, J.; Tomer, K. B.; Ball, L. M., A 2-iminohydantoin from the oxidation of guanine. Chemical Research in Toxicology 2006, 19 (4), 506-510.

(47) Ferris, T. D.; Lee, P. T.; Farrar, T. C., Synthesis of propiolamide and H-1, C-13 and N-15 NMR spectra of formamide, acetamide and propiolamide. Magnetic Resonance in Chemistry 1997, 35 (9), 571-576.

(48) Enami, S.; Hoffmann, M. R.; Colussi, A. J., Ozonolysis of uric acid at the air/water interface. Journal of Physical Chemistry B 2008, 112 (14), 4153-4156.

(49) Motta, F. D.; Miyamoto, S.; Martinez, G. R.; Gennari Medeiros, M. H.; Di Mascio, P., Ozone Reacts With 2 '-Deoxyguanosine and Polyamines Generating Singlet Molecular Oxygen. Free Radical Biology and Medicine 2008, 45, S20-S21.

(50) Munoz, F.; Mvula, E.; Braslavsky, S. E.; von Sonntag, C., Singlet dioxygen formation in ozone reactions in aqueous solution. J. Chem. Soc.-Perkin Trans. 2 2001, (7), 1109-1116.

(51) Vialas, C.; Pratviel, G.; Claparols, C.; Meunier, B., Efficient oxidation of 2 '-deoxyguanosine by Mn-TMPyP/KHSO(5) to imidazolone dIz without formation of 8-oxo-dG. Journal of the American Chemical Society 1998, 120 (45), 11548-11553.

(52) Buffle, M.-O.; Schumacher, J.; Meylan, S.; Jekel, M.; von Gunten, U., Ozonation and advanced oxidation of wastewater: Effect of O-3 dose, pH, DOM and HO center dot-scavengers on ozone decomposition and HO center dot generation. Ozone-Science & Engineering 2006, 28 (4), 247-259.

(53) Buffle, M. O.; Von Gunten, U., Phenols and amine induced HO center dot generation during the initial phase of natural water ozonation. Environ. Sci. Technol. 2006, 40 (9), 3057-3063.

(54) Feron, V. J.; Til, H. P.; Devrijer, F.; Woutersen, R. A.; Cassee, F. R.; Vanbladeren, P. J., Aldehydes - Occurrence, Carcinogenic Potential, Mechanism of Action and Risk Assessment. Mutation Research 1991, 259 (3-4), 363-385.

(55) O'Brien, P. J.; Siraki, A. G.; Shangari, N., Aldehyde sources, metabolism, molecular toxicity mechanisms, and possible effects on human health. Crit. Rev. Toxicol. 2005, 35 (7), 609-662.

(56) Alvares, A. B. C.; Diaper, C.; Parsons, S. A., Partial oxidation by ozone to remove recalcitrance from wastewaters - A review. Environmental Technology 2001, 22 (4), 409-427.

(57) Beltran, F. J.; Garcia-Araya, J. F.; Alvarez, P. M., Integration of continuous biological and chemical (ozone) treatment of domestic wastewater: 2. Ozonation followed by biological oxidation. Journal of Chemical Technology and Biotechnology 1999, 74 (9), 884-890.

(58) Sangave, P. C.; Gogate, P. R.; Pandit, A. B., Combination of ozonation with conventional aerobic oxidation for distillery wastewater treatment. Chemosphere 2007, 68 (1), 32-41.

(59) Takahashi, N.; Kumagai, T., Removal of dissolved organic carbon and color from dyeing wastewater by pre-ozonation and subsequent biological treatment. Ozone-Science & Engineering 2006, 28 (4), 199-205.

6 FINAL CONCLUSIONS

The results obtained in this thesis clearly demonstrate that antiviral drugs are a new class of pharmaceuticals which are of environmental concern. The developed **highly sensitive analytical method** enabled the determination of antiviral drugs at concentrations in the low ng/L-range. This allowed, for the first time, the detection of a broad spectrum of antiviral drugs in the aquatic environment.

Considering the main entrance pathways of antiviral drugs into the urban water cycle, excretions via urine or faeces from treated individuals play a dominant role. Therefore, WWTPs represent the first potential barrier for the removal of these class of pharmaceuticals. However, the results revealed an **incomplete removal** of most antiviral drugs **in conventional wastewater treatment** leading to their emission into receiving waters. Consequently, highest concentrations were observed in surface waters with high proportions of treated wastewater.

The monitoring of antiviral drugs in the river Rhine revealed that **industrial discharges from manufacturers** can contribute significantly to the emission of anti-influenza drugs such as oseltamivir into surface waters. Individual sources could be distinguished by the simultaneous analysis of oseltamivir and its active human metabolite oseltamivir carboxylate. It was shown that elevated quantities of anti-influenza drugs are emitted into surface waters during flu epidemics attributable to the incomplete removal of anti-influenza drugs in wastewater treatment.

The **fate of antiviral drugs in wastewater treatment** was investigated for acyclovir and penciclovir. The results obtained highlight that their degradation is in general not leading to a complete mineralization but rather results in the formation of transformation products (TPs). It was shown that the formed TPs often feature only slight structural changes compared to the parent compounds, indicating a similar pharmaceutical activity but a higher polarity. Furthermore, it was demonstrated that already slight structural differences in the chemical structure of organic compounds are decisive for their degradation behavior.

The **identification of TPs** is highly challenging. The results clearly highlight the necessity of using different independent analytical techniques such as high-resolution mass spectrometry (HR-MS) and NMR to identify TPs. HR-MS alone was in most cases only capable of proposing tentative chemical structures even for TPs which show slight structural modifications compared to the parent antiviral drug. The isolation of individual TPs thereby also allows for their quantification in environmental samples and for the calculation of mass balances. This is crucial to assess whether all environmentally relevant TPs have been identified and to follow the fate of TPs in the environment.

For acyclovir it was shown that the **transformation in wastewater treatment** leads to the formation of a stable TP which was identified as carboxy-acyclovir. Due to its persistence, carboxy-acyclovir was detected in wastewater treatment plant effluents and in surface waters. Furthermore, it was also detected in groundwater highlighting its high persistence in the aquatic environment. Furthermore, the incomplete removal in conventional drinking water treatment via bank and sand filtration leads to the presence of carboxy-acyclovir also in finished drinking water.

Advanced water treatment via **ozonation** was shown to efficiently eliminate acyclovir and its transformation product carboxy-acyclovir. However, under conventional treatment conditions the **formation of an oxidation product** (OP) was observed which was identified using HR-MS and NMR. The recalcitrance of the OP in subsequent biological treatment shows that ozonation does not necessarily lead to compounds prone to microbial attack. Furthermore, a bacterial toxicity was observed for the OP. Due to the presence of carboxy-acyclovir in the feed water of a waterworks, the OP was detected in finished drinking water after ozonation and subsequent activated carbon treatment.

Therefore, this is one of the first studies in which the fate of a pharmaceutical compound was followed through almost the complete water cycle, from the release into the sewer system to advanced treatment via ozone in a waterworks.
In summary, the following overall conclusions can be drawn:

A) Antiviral drugs are ubiquitously present in the aquatic environment.

B) TPs formed in WWTPs can be stable and even occur in drinking water.

C) Biological TPs formed in WWTPs can be further transformed during oxidative water treatment using ozone leading to stable oxidation products

D) Oxidation products formed during ozonation can be toxic for bacteria due to the formation of reactive moieties such as formaldehydes.

APPENDIX

A.1 SUPPORTING INFORMATION OF CHAPTER 2

Antiviral Drugs in Wastewater and Surface Waters: A new Class of Pharmaceuticals of Environmental Relevance?

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Content

Pages: 14 Tables:8 Figures: 6

Tables

Figures

Reagents and Chemicals

Investigated substances and respective surrogate standards were obtained from the following suppliers: acyclovir from Sigma-Aldrich (Taufkrichen, Germany), penciclovir from VWR international (Darmstadt, Germany) and lamivudine, nevirapine (anhydrous), ribavirin, stavudine, zidovudine from LGC Standards (Wesel, Germany). Abacavir (hemisulfate) was kindly provided by GlaxoSmithKline (München, Germany), oseltamivir (phosphate) and oseltamivir carboxylate (tartrate) by Roche Diagnostics (Mannheim, Germany). Surrogate standards (acyclovir-d_4; $^{13}C,^{15}N_2$-lamivudine, nevirapine-d_5; penciclovir-d_4; ribavirin-$^{13}C_5$; stavudine-d_3; zidovudine-d_3 (isotopic purity > 99 %)) were obtained from Campro Scientific (Berlin, Germany). All solvents were LC-grade and were obtained from LGC Standards (Wesel, Germany), except for dimethyl sulfoxide (DMSO) which was obtained from VWR international (Darmstadt, Germany). MilliQ water was provided by a water purification system from Millipore (Bedford, MA, USA). Groundwater was taken from a deep well in Ahrenberg, Koblenz (Germany) which is not influenced by anthropogenic contamination. Hydrochloric acid, ammonium acetate, ammonium hydroxide, and ammonium formate were purchased from Carl Roth (Karlsruhe, Germany).

Individual stock solutions of analytes (1 mg mL^{-1}) were prepared in DMSO (acyclovir, penciclovir, lamivudine, nevirapine) and methanol (abacavir, oseltamivir, oseltamivir carboxylate, ribavirin, stavudine, zidovudine). Stock solutions of internal standards at 0.1 mg mL^{-1} were dissolved in the same solvents as respective analytes. A standard solution of all target analytes and a standard solution of all surrogate standards was prepared at a concentration of 10 µg mL^{-1} in HPLC-H_2O/methanol 90/10 (v/v) for RPLC and in acetonitrile/H_2O 90/10 (v/v) for HILIC, and subsequently diluted as necessary.

SPE method development

For method development purposes different SPE sorbent materials were tested for their efficiency to extract antiviral drugs from aqueous matrices. Based on the chemical properties of the analytes two types of sorbent materials were investigated: cation exchangers (as most of the analytes are positively charged at low pH) and polymers which in general show good extraction efficiencies for polar substances. The extraction efficiencies of the following SPE cartridges was assessed: Oasis MCX (60 mg/3 mL), Oasis HLB (200 mg/6 mL) from Waters (Eschborn, Germany); Plexa PCX (500 mg/6 mL), Bond Elut ENV (500 mg/3 mL), Bond Elut PPL (500 mg/3 mL) from Varian (Darmstadt, Germany); Strata-X (200 mg/6 mL), SDB-L (500 mg/6 mL), Strata SCX (200 mg/6 mL) from Phenomenex (Aschaffenburg, Germany); Supelclean ENVI-Carb Plus (400 mg/ 1 mL) from Supelco (Schnelldorf, Germany); and Isolute ENV^+ (200 mg/3 mL and 500 mg/6 mL) from Biotage (Upsala, Sweden). Conditioning and elution of analytes was performed as proposed by the manufacturers except for Supelclean ENVI-Carb Plus cartridges which were eluted successively with methanol,

acetone and MTBE (with and without the addition of formic acid) to determine whether desorption of analytes from the sorbent material can be achieved. pH values were adjusted individually for each cartridge type to obtain maximum recoveries of analytes depending on the predominant sorption mechanisms. For cation exchanger (Oasis MCX, Plexa PCX, Strata SCX) pH values were adjusted to pH 1, 2 and 3, for styrene divinylbenzene polymers to pH 3, 5 and 7 (Strata-X) and 7, 8.5, 10 (Bond Elut ENV, Bond Elut PPL and Isolute ENV$^+$). For the hydrophilic-lipophilic sorbent (Oasis HLB) pH was adjusted to 3, 5 and 7. Supelclean ENVI-Carb Plus cartridges were only tested at pH 8. Tests were carried out in triplicates using 250 mL of groundwater. Analytes were added to obtain a concentration of 500 ng mL^{-1} in the final extract. Absolute recoveries were calculated by comparing peak areas of spiked samples with an external standard containing the same amount of analytes.

HILIC method

A 3 µm Luna HILIC (150 mm x 3 mm) column protected by SecurityGuard column (4 x 3 mm i.d.; Phenomenex, Aschaffenburg, Germany) was used. Acetonitrile/5 mM NH4-formate, pH 3.2 90/10 (A) and acetonitrile/5 mM NH4-formate, pH 3.2 70/30 (B) were used as mobile phases. Chromatographic separation was achieved as described in the following: 0-2.5 min, 100 % A; 2.5-10 min, 100 % B; 10-15 min, 100 % A. Analysis run time was 15 min. As for RPLC, MRM transitions were measured in time periods: In period 1 (0–5.0 min), acyclovir, lamivudine, nevirapine, oseltamivir, stavudine and zidovudine were measured with a dwell time of 50 ms and the following ESI source parameters were used: CUR, 15 psi; GS1, 20 psi; GS2, 10 psi. In period 2 (5.0–15.0 min) penciclovir and oseltamivir carboxylate were measured with a dwell time of 250 ms and ESI source parameters were adjusted to: CUR, 35 psi; GS1, 45 psi; GS2, 45 psi.
Final sample extracts were evaporated till dryness and reconstituted in 1 mL acetonitrile/5 mM NH_4-formate (pH 3.2) 90/10 before analysis.

Optimization of sample volumes

For determination of optimal samples volumes, 100, 250 and 500 mL of WWTP influents and effluents and 100, 250, 500 and 1000 mL of groundwater were extracted in triplicate using the above described SPE method. Analytes were added to obtain a concentration of 500 ng mL^{-1} in the final extract. To determine whether reduction in recoveries are caused by ion suppression/enhancement during ESI-MS/MS or inappropriate retention during SPE, samples were either spiked prior to SPE or afterwards. For WWTP influent and effluent non-spiked samples were used to correct for blank concentrations. RPLC was used for chromatographic separation.

Table S1. Precursor, product ions, retention times and respective periods in LC/MS/MS detection[a]

Substance	retention time in min (period no.)		MRM 1	MRM 2	DP [V]	CE (MRM1 /MRM2) [eV]	CXP (MRM1 /MRM2) [V]
	Hydro-RP	Luna HILIC					
Analytes							
Ribavirin	3.35 (1)	3.74 (1)	245.0 → 112.9	245.0 → 95.9	41	8 / 4	11 / 43
Acyclovir	9.44 (2)	4.35 (1)	226.1 → 152.0	226.1 → 135.0	71	12 / 14	17 / 43
Penciclovir	9.71 (2)	5.74 (2)	254.1 → 152.0	254.1 → 135.0	91	10 / 12	25 / 45
Lamivudine	9.95 (2)	3.32 (1)	230.5 → 112.0	230.5 → 95.0	56	18 / 0	19 / 51
Stavudine	10.20 (2)	2.60 (1)	225.3 → 126.9	225.3 → 69.2	16	4 / 2	11 / 29
Oseltamivir carboxylate	10.78 (3)	7.98 (2)	285.2 → 197.2	285.2 → 138.2	46	16 / 10	13 / 27
Zidovudine	10.89 (3)	2.45 (1)	268.1 → 127.0	268.1 → 110.0	31	10 / 6	17 / 47
Abacavir	11.42 (3)	2.99 (1)	287.1 → 191.4	287.1 → 79.1	31	30 / 12	25 / 47
Nevirapine	11.73 (3)	2.40 (1)	267.1 → 226.1	267.1 → 80.2	91	18 / 4	37 / 63
Oseltamivir	12.35 (3)	3.71 (1)	313.3 → 225.1	313.3 → 166.2	51	6 / 12	15 / 29
Internal Standards							
Ribavirin-$^{13}C_5$	3.30 (1)	3.71 (1)	250.3 → 113.1	250.3 → 96.0	46	8 / 6	19 / 49
Acyclovir-d_4	9.38 (2)	4.34 (1)	230.3 → 152.0	230.3 → 135.1	46	12 / 10	19 / 41
Penciclovir-d_4	9.68 (2)	5.73 (2)	258.3 → 152.1	258.3 → 135.1	66	0 / 8	27 / 49
^{13}C-$^{15}N_2$-Lamivudine	9.96 (2)	3.31 (1)	233.2 → 115.1	233.2 → 97.0	61	8 / 6	17 / 55
Stavudine-d_3	10.21 (2)	2.57 (1)	228.3 → 130.1	228.3 → 113.1	26	8 / 8	17 / 39
Zidovudine-d_3	10.89 (3)	2.41 (1)	271.3 → 130.0	271.3 → 113.1	31	10 / 6	15 / 45
Nevirapine-d_5	10.71 (3)	2.40 (1)	272.3 → 227.1	272.3 → 198.1	71	18 / 14	37 / 55

[a] DP = Declustering potential; CE = Collision energy; CXP = Collision cell exit potential

Table S2. Absolute recoveries and 95% confidence intervals of antiviral drugs on tested SPE sorbent materials using 250 mL of groundwater. Only the pH for which highest recoveries were obtained is shown (n = 3).

Analyte	Isolute ENV+ (500 mg) pH 8	Isolute ENV+ (200 mg) pH 8	SDB-L, pH 8	ENVI-Carb Plus pH 8	Bond Elut ENV pH 8	Bond Elut PPL pH 7	Oasis HLB pH 3	Oasis MCX pH 3	Strata-X pH 3	Strata SCX pH 2	Plexa PCX pH 2
RBV	66 ± 4	59 ± 1	2.0 ± 0.1	120 ± 32	1.1 ± 0.5	< 1	< 1	< 1	< 1	< 1	1.7 ± 0.8
ACV	119 ± 27	78 ± 10	5.3 ± 0.6	15 ± 1	12 ± 1	1.1 ± 0.2	2.4 ± 0.4	10 ± 2	< 1	2.6 ± 0.6	61 ± 31
PCV	110 ± 9	81 ± 8	7.8 ± 1.3	10 ± 6	27 ± 6	1.8 ± 0.4	2.7 ± 0.9	30 ± 9	< 1	3.1 ± 1.5	48 ± 23
3TC	76 ± 15	n.a.	24 ± 2	56 ± 13	39 ± 5	6.6 ± 1.4	n.a.	n.a.	n.a.	n.a.	n.a.
d4T	115 ± 38	n.a.	74 ± 7	93 ± 21	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
ABC	89 ± 11	n.a.	72 ± 8	24 ± 5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
NVP	106 ± 6	n.a.	75 ± 6	85 ± 25	82 ± 4	71 ± 17	n.a.	n.a.	n.a.	n.a.	n.a.
OP	95 ± 11	19 ± 3	76 ± 1	33 ± 5	70 ± 14	66 ± 8	105 ± 17	67 ± 2	101 ± 10	25 ± 5	84 ± 9
OC	70 ± 8	84 ± 1	25 ± 3	20 ± 16	51 ± 7	11 ± 1	53 ± 14	95 ± 10	23 ± 1	32 ± 5	92 ± 4
ZDV	103 ± 23	n.a.	102 ± 7	58 ± 12	102 ± 9	103 ± 3	n.a.	n.a.	n.a.	n.a.	n.a.

n.a. not analysed

Table S3. S/N ratios and 95 % confidence intervals of the analytes in the investigated matrices. The respective concentrations are given in parenthesis (spiked samples, n = 5; samples where background concentrations were used, n = 3).

WWTP Effluent	**RBV** (5.0 ng)	**ACV** (3.2 ng)	**PCV** (5.0 ng)	**3TC** (5.0 ng)	**d4T** (5.0 ng)	**ABC** (5.0 ng)	**NVP** (1.7 ng)	**OP** (15 ng)	**OC** (8.6 ng)	**ZDV** (31 ng)
S/N MRM 1	37 ± 7	24 ± 4	60 ± 6	< LOQ	34 ± 9	140 ± 14	120 ± 18	180 ± 33	61 ± 7	230 ± 26
S/N MRM 2	23 ± 8	7 ± 3	13 ± 2	< LOQ	6 ± 1	32 ± 4	39 ± 3	170 ± 28	33 ± 2	60 ± 9
WWTP Influent	**RBV** (5.0 ng)	**ACV** (190 ng)	**PCV** (3.3 ng)	**3TC** (21 ng)	**d4T** (1.1 ng)	**ABC** (15 ng)	**NVP** (0.6 ng)	**OP** (4.0 ng)	**OC** (7.1 ng)	**ZDV** (30 ng)
S/N MRM 1	71 ± 7	1000 ± 11	30 ± 3	150 ± 2	14 ± 11	85 ± 5	55 ± 16	48 ± 21	47 ± 5	190 ± 44
S/N MRM 2	12 ± 3	250 ± 33	17 ± 3	7 ± 3	15 ± 9	31 ± 2	15 ± 2	11 ± 2	13 ± 7	27 ± 4
Surface water	**RBV** (5.0 ng)	**ACV** (1.2 ng)	**PCV** (5.0 ng)	**3TC** (5.0 ng)	**d4T** (5.0 ng)	**ABC** (5.0 ng)	**NVP** (0.5 ng)	**OP** (8.9 ng)	**OC** (0.9 ng)	**ZDV** (1.7 ng)
S/N MRM 1	35 ± 5	31 ± 5	160 ± 41	220 ± 38	210 ± 53	360 ± 34	55 ± 7	220 ± 33	20 ± 2	25 ± 18
S/N MRM 2	9 ± 1	7 ± 3	40 ± 10	7 ± 2	18 ± 1	92 ± 7	6 ± 2	210 ± 28	12 ± 7	7 ± 3

Table S4. Coordinates of sampling locations in the Hessisches Ried region and along the Ruhr river.

river location	GPS coordinates	comment
	Hessian Ried	
HR_01	N 50° 00.031' E 008° 19.352'	Main
HR_02	N 49° 57.724' E 008° 20.893'	Schwarzbach
HR_03	N 49° 54.857' E 008° 24.599'	Landgraben
HR_04	N 49° 58.643 'E 008° 30.395'	Gundbach
HR_05	N 49° 59.948' E 008° 24.236'	Wickerbach
HR_06	N 50° 05.095' E 008° 37.300'	WWTP Frankfurt
HR_07	N 50° 05.290' E 008° 37.792'	Main before discharge of WWTP
HR_08	N 50° 05.279' E 008° 37.640'	Main after discharge of WWTP
HR_09	N 50° 06.724' E 008° 49.921'	Bieber
HR_10	N 50° 06.587' E 008° 50.401'	Rodau
HR_11	N 50° 11.395' E 008° 43.256'	Erlenbach
HR_12	N 50° 10.912' E 008° 41.833'	Eschbach
	Ruhr catchment	
Ru_01	N 51° 15.468' E 008° 31.760'	Ruhr near spring
Ru_02	N 51° 16.723' E 008° 30.991'	Ruhr after WWTP Niedersfeld
Ru_03	N 51° 18.356' E 008° 30.165'	Ruhr
Ru_04	N 51° 19.881' E 008° 29.300'	Ruhr after Neger
Ru_05	N 51° 19.753' E 008° 29.232'	Neger
Ru_06	N 51° 21.488' E 008° 22.955'	Ruhr after Valme
Ru_07	N 51° 21.119' E 008° 20.326'	Ruhr after WWTP Bestwig-Velmede
Ru_08	N 51° 20.777' E 008° 12.305'	Ruhr before Wenne
Ru_09	N 51° 20.026' E 008° 09.780'	Wenne
Ru_10	N 51° 21.825' E 008° 10.270'	Ruhr after Wenne
Ru_11	N 51° 23.426' E 008° 07.846'	Ruhr
Ru_12	N 51° 25.237' E 008° 01.371'	Ruhr after WWTP Arnsberg
Ru_13	N 51° 25.890' E 007° 59.376'	Röhr
Ru_14	N 51° 27.335' E 007° 57.490'	Möhne
Ru_15	N 51° 27.349' E 007° 57.238'	Ruhr before Möhne
Ru_16	N 51° 27.502' E 007° 57.324'	Ruhr after Möhne
Ru_17	N 51° 29.265' E 007° 55.450'	Ruhr after WWTP Neheim
Ru_18	N 51° 27.971' E 007° 39.696'	Ruhr
Ru_19	N 51° 24.792' E 007° 30.793'	Ruhr before Lenne
Ru_20	N 51° 24.497' E 007° 29.596'	Lenne
Ru_21	N 51° 25.073' E 007° 28.560'	Ruhr after Lenne / Hengsteneysee
Ru_22	N 51° 23.086' E 007° 26.976'	Volme
Ru_23	N 51° 22.988' E 007° 24.276'	Ruhr after Volme
Ru_24	N 51° 26.263' E 007° 16.948'	Effluent WWTP Bochum-Ölbachtal
Ru_25	N 51° 24.673' E 007° 14.836'	Effluent Kemnader reservoir
Ru_26	N 51° 24.773' E 007° 10.990'	Ruhr
Ru_27	N 51° 23.430' E 007° 09.228'	Hattingen after WWTP Hattingen
Ru_28	N 51° 23.816' E 007° 00.196'	Baldeneysee
Ru_29	N 51° 21.566' E 006° 56.402'	Ruhr
Ru_30	N 51° 26.532' E 006° 49.521'	Ruhr, Duisburg before harbour
Rh_01	N 51° 26.141' E 006° 42.937'	Rhine before conjunction with Ruhr
Rh_02	N 51° 27.651' E 006° 43.525'	Rhine after conjunction with Ruhr
Rh_03	N 51° 33.562' E 006° 41.338'	Rhine, Dinslaken before Emscher
Em_01	N 51° 33.753' E 006° 41.522'	Mouth of Emscher

Table S5. WWTPs in the catchment area of the Ruhr and the Emscher (1).

sampling point	comment	WWTP			
		name	population equivalents (PE)	affiliated citizens	wastewater discharge [L Person^{-1} d^{-1}]
Ru_02	Ruhr after WWTP Niedersfeld	Winterberg-Niedersfeld	4,950	4,087	2,034.30
Ru_05	Neger		No data available		
Ru_06	Ruhr after Valme		No data available		
Ru_07	Ruhr after WWTP Bestwig-Velmede	Bestwig-Velmede	17,500	33,863	1,226.00
Ru_08	Ruhr after Wenne		No data available		
Ru_12	Ruhr after WWTP Arnsberg	Arnsberg	64,000	24,389	458.2
		Arnsberg-Neheim	75,000	119,034	312.8
		Arnsberg-Wildshausen	65,000	107,280	359
			204,000	**250,703**	**1,130**
Ru_13	Röhr	Sundern	16,000	19,610	672
Ru_14	Möhne	Möhnesee-Völlinghausen	25,000	11,803	517.9
		Warstein-Belecke	16,000	9,864	897.2
		Brilon-Bontkirchen	1,100	516	491.5
		Brilon-Madfeld	3,000	1,388	884.3
		Brilon-Messinghausen	6,000	2,110	428.4
		Brilon-Petersborn-Gudenhagen	4,500	1,795	1,045.10
			55,600	**27,476**	**4,264**
Ru_17	Ruhr after WWTP	Arnsberg-Neheim	75,000	119,034	312.8
Ru_20	Lenne	Hagen Fley	69,760	50,359	330
		Iserlohn Letmathe	70,000	59,604	355.5
		Altena	35,000	24,467	580.9
		Werdohl	35,000	23,734	718.3
		Plettenberg	34,000	31,393	762.9
		Finnentrop	26,700	25,066	506.4
		Lennestadt Grevenbrück	41,300	24,879	715.4
		Lennestadt	38,150	32,648	1,003.10
		Schmallenberg	15,000	10,233	939.2
			364,910	**282,383**	**5,912**
Ru_23	Ruhr after Volme	Schalksmühle	29,000	23,435	687.7
		Volmetal	33,500	33,782	376.5
		Kierspe Bahnhof	14,000	9,582	551.8
		Meinerzhagen	18,000	15,370	573.1
			94,500	**82,169**	**2,189**
Ru_24	Ruhr after WWTP Bochum-Ölbachtal	Bochum-Oelbachtal	300,000	167,173	148.7
Ru_27	Ruhr after WWTP Hattingen	Hattingen	100,000	78,790	378.1
			1,232,460	**1,065,288**	**17,256**
Em_01	Emscher	Emschermündung	2,400,000	2,772,239	464.9
		Bottrop	1,340,000	1,100,693	280.9
		Dortmund-Nord	625,000	645,917	283.7
			4,365,000	**4,518,849**	**1,030**

Table S6. Mean concentrations of antiviral drugs in samples taken along the Ruhr river. Absolute deviations of the two samples taken at each location are given in parenthesis (n = 2). Ribavirin, lamivudine and penciclovir are not shown as they were absent in all samples

sampling location	acyclovir	stavudine	abacavir	nevirapine	oseltamivir	oseltamivir carboxylate	zidovudine
Ru_01	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ru_02	4.5 (0.1)	2.6 (0.1)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ru_03	1.9 (0.1)	2.1 (0.1)	0.5 (0.1)	< LOQ	< LOQ	< LOQ	< LOQ
Ru_04	< LOQ	1.7 (0.2)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ru_05	< LOQ	1.6 (0.1)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ru_06	< LOQ	1.3 (0.1)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ru_07	2.6 (0.1)	< LOQ	< LOQ	< LOQ	0.8 (0.1)	1.3 (0.2)	2.1 (0.1)
Ru_08	1.1 (0.1)	< LOQ	< LOQ	< LOQ	< LOQ	0.4 (0.1)	< LOQ
Ru_09	3.5 (0.1)	2.4 (0.1)	< LOQ	< LOQ	0.3 (0.1)	0.8 (0.1)	3.1 (0.1)
Ru_10	1.3 (0.1)	< LOQ	< LOQ	< LOQ	0.2 (0.1)	0.5 (0.1)	< LOQ
Ru_11	4.1 (0.1)	< LOQ	< LOQ	< LOQ	0.2 (0.1)	0.6 (0.2)	1.2 (0.1)
Ru_12	6.2 (0.1)	< LOQ	< LOQ	< LOQ	0.3 (0.1)	0.9 (0.1)	1.1 (0.2)
Ru_13	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	1.8 (0.1)
Ru_14	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ru_15	4.3 (0.2)	< LOQ	< LOQ	< LOQ	0.3 (0.1)	0.7 (0.2)	1.4 (0.1)
Ru_16	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ru_17	3.7 (0.2)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ru_18	7.1 (0.2)	< LOQ	< LOQ	< LOQ	0.4 (0.1)	< LOQ	2.2 (0.1)
Ru_19	8.5 (0.3)	< LOQ	< LOQ	< LOQ	0.5 (0.1)	0.8 (0.3)	3.5 (0.1)
Ru_20	20.1 (0.6)	3.1 (0.1)	< LOQ	< LOQ	0.6 (0.1)	1.4 (0.2)	6.2 (0.1)
Ru_21	8.3 (0.1)	< LOQ	< LOQ	< LOQ	0.7 (0.2)	1.0 (0.2)	3.5 (0.1)
Ru_22	6.1 (0.1)	4.3 (0.2)	< LOQ	< LOQ	1.0 (0.1)	0.8 (0.3)	4.8 (0.2)
Ru_23	8.9 (0.1)	< LOQ	< LOQ	< LOQ	0.6 (0.1)	< LOQ	7.6 (0.1)
Ru_24	10.2 (0.3)	< LOQ	< LOQ	3.8 (0.1)	2.2 (0.2)	1.4 (0.1)	29 (0.4)
Ru_25	6.6 (0.4)	2.9 (0.3)	< LOQ	< LOQ	0.8 (0.1)	0.7 (0.1)	6.0 (0.1)
Ru_26	6.7 (0.2)	3.6 (0.1)	< LOQ	< LOQ	0.8 (0.1)	< LOQ	5.8 (0.1)
Ru_27	6.4 (0.1)	< LOQ	< LOQ	< LOQ	1.0 (0.2)	0.9 (0.2)	6.2 (0.1)
Ru_28	6.5 (0.1)	< LOQ	< LOQ	< LOQ	0.8 (0.1)	0.8 (0.2)	6.8 (0.2)
Ru_29	6.0 (0.1)	< LOQ	< LOQ	< LOQ	0.9 (0.1)	0.9 (0.1)	7.0 (0.1)
Ru_30	7.0 (0.4)	2.2 (0.3)	< LOQ	< LOQ	0.9 (0.1)	0.6 (0.1)	7.3 (0.2)
Rh_01	3.6 (0.1)	< LOQ	< LOQ	< LOQ	17 (1)	1.3 (0.1)	5.0 (0.2)
Rh_02	4.2 (0.1)	< LOQ	< LOQ	< LOQ	15 (2)	1.1 (0.1)	5.8 (0.2)
Rh_03	3.4 (0.2)	< LOQ	< LOQ	< LOQ	15 (3)	1.1 (0.1)	4.8 (0.2)
Em_01	30 (0.5)	< LOQ	< LOQ	3.2 (0.1)	2.9 (0.3)	2.4 (0.3)	94 (5)

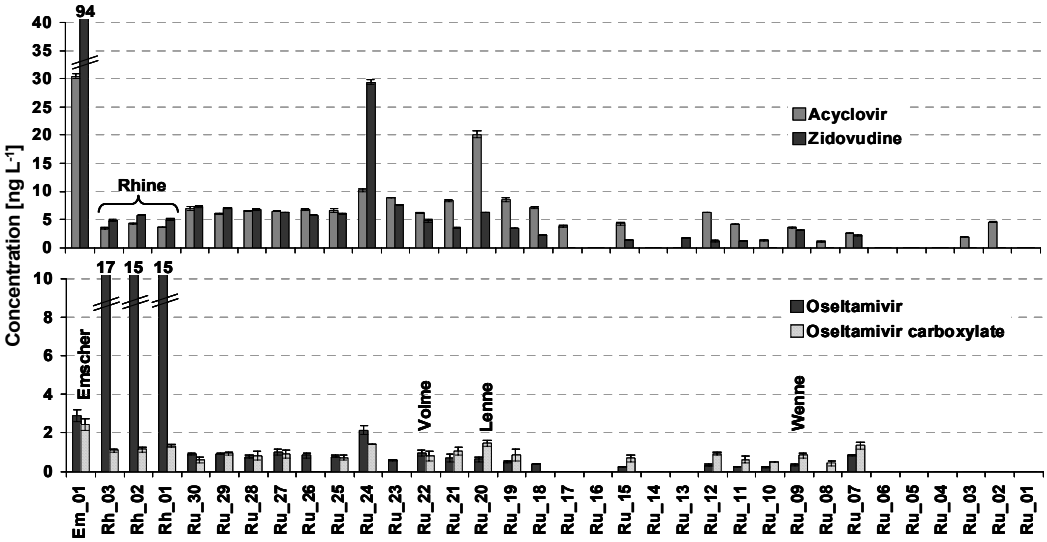

Table S7. OP/OC ratios in sampled locations.

sampling location	OP/OC	sampling location	OP/OC
Ruhr[a]		**Emscher**[a]	
Ru_01	n.a.	Em_01	1.19 ± 0.12
Ru_02	n.a.		
Ru_03	n.a.	**Rhine**[a]	
Ru_04	n.a.	Rh_01	12.7 ± 0.3
Ru_05	n.a.	Rh_02	13.0 ± 3.3
Ru_06	n.a.	Rh_03	13.8 ± 3.5
Ru_07	0.62 ± 0.11	Koblenz[b]	12.4 ± 1.8
Ru_08	n.a.	Weil am Rhein	13.1
Ru_09	0.41 ± 0.12		
Ru_10	0.44 ± 0.01	**Hessian Ried**[b]	
Ru_11	0.40 ± 0.11	HR_01	n.a.
Ru_12	0.34 ± 0.08	HR_02	3.10 ± 0.7
Ru_13	n.a.	HR_03	2.04 ± 0.45
Ru_14	n.a.	HR_04	1.11 ± 0.73
Ru_15	0.37 ± 0.10	HR_05	0.73 ± 0.11
Ru_16	n.a.	HR_07	n.a.
Ru_17	n.a.	HR_08	1.92 ± 1.08
Ru_18	n.a.	HR_09	n.a.
Ru_19	0.57 ± 0.29	HR_10	3.05 ± 1.12
Ru_20	0.44 ± 0.05	HR_11	0.57 ± 0.17
Ru_21	0.66 ± 0.04	HR_12	1.27 ± 0.13
Ru_22	1.17 ± 0.59		
Ru_23	n.a.	**raw wastewater**[b]	
Ru_24	1.51 ± 0.16	WWTP1	0.28 ± 0.11
Ru_25	1.08 ± 0.25	WWTP2	n.a.
Ru_26	n.a.		
Ru_27	1.11 ± 0.38	**treated wastewater**[b]	
Ru_28	0.95 ± 0.38	WWTP1	0.91 ± 0.20
Ru_29	0.98 ± 0.10	WWTP2	0.72 ± 0.23
Ru_30	1.48 ± 0.51		

n.a. not available. [a] given as mean values ± absolute deviations from means (n = 2). [b] given as mean values ± 95% confidence intervals (n = 3).

Table S8. Measured and predicted loads for oseltamivir and its metabolite oseltamivir carboxylate and zidovudine.

sampling location	sum of oseltamivir and oseltamivir carboxylate		zidovudine	
	measured load [g d^{-1}]	predicted load [g d^{-1}][a]	measured load [g d^{-1}]	predicted load [g d^{-1}][a,b]
Ru_07	0.7	0.1	0.7	0.4
Ru_12	0.5	0.6	0.5	3.3
Ru_18	0.4	0.9	2.7	5.2
Ru_23	1.2	1.6	14.1	9.3
Ru_27	3.8	2.1	12.5	12.1
Lenne	2.0	0.6	6.1	3.2
Volme	0.2	0.2	0.5	0.9

[a] predicted from the total annual prescribed amount (2) and the number of affiliated citizens of WWTPs upstream of the sampled location (Table S6)

[b] taking into account that only 20% are excreted as parent compound and assuming a negligible removal during wastewater treatment (3)

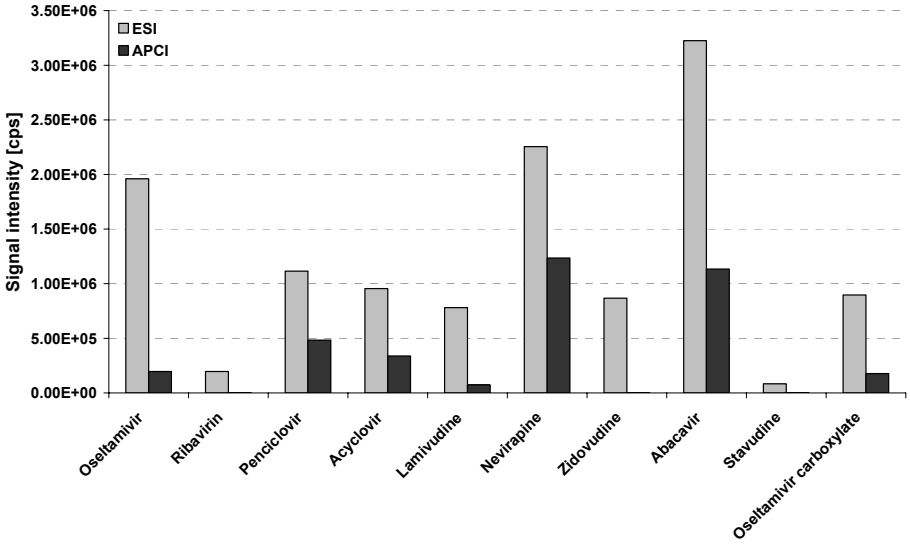

Figure S1. Signal intensities of analytes for ESI and APCI using a standard mixture (500 ng mL^{-1}). Standard deviations are given as error bars (n = 3).

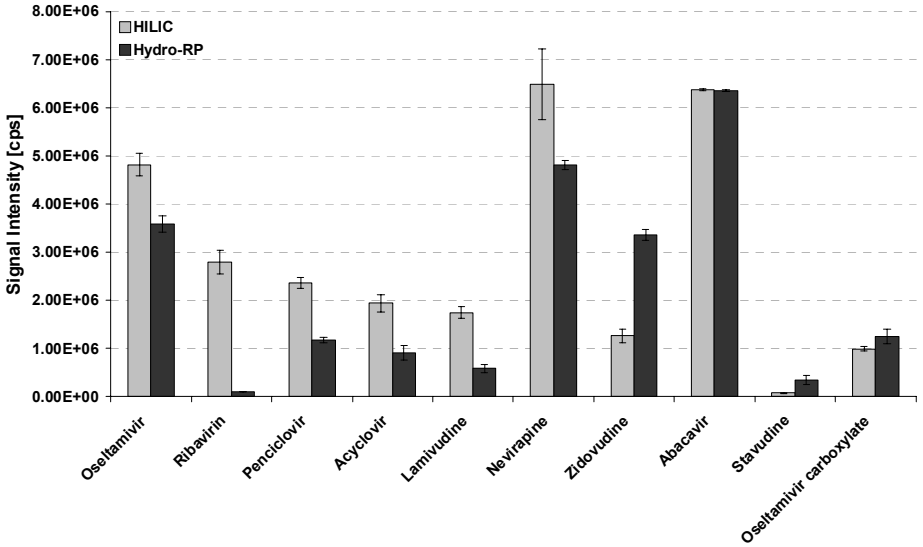

Figure S2. Signal intensities of analytes for both investigated chromatographic methods using a standard mixture (500 ng mL^{-1}). Standard deviations are given as error bars (n = 3).

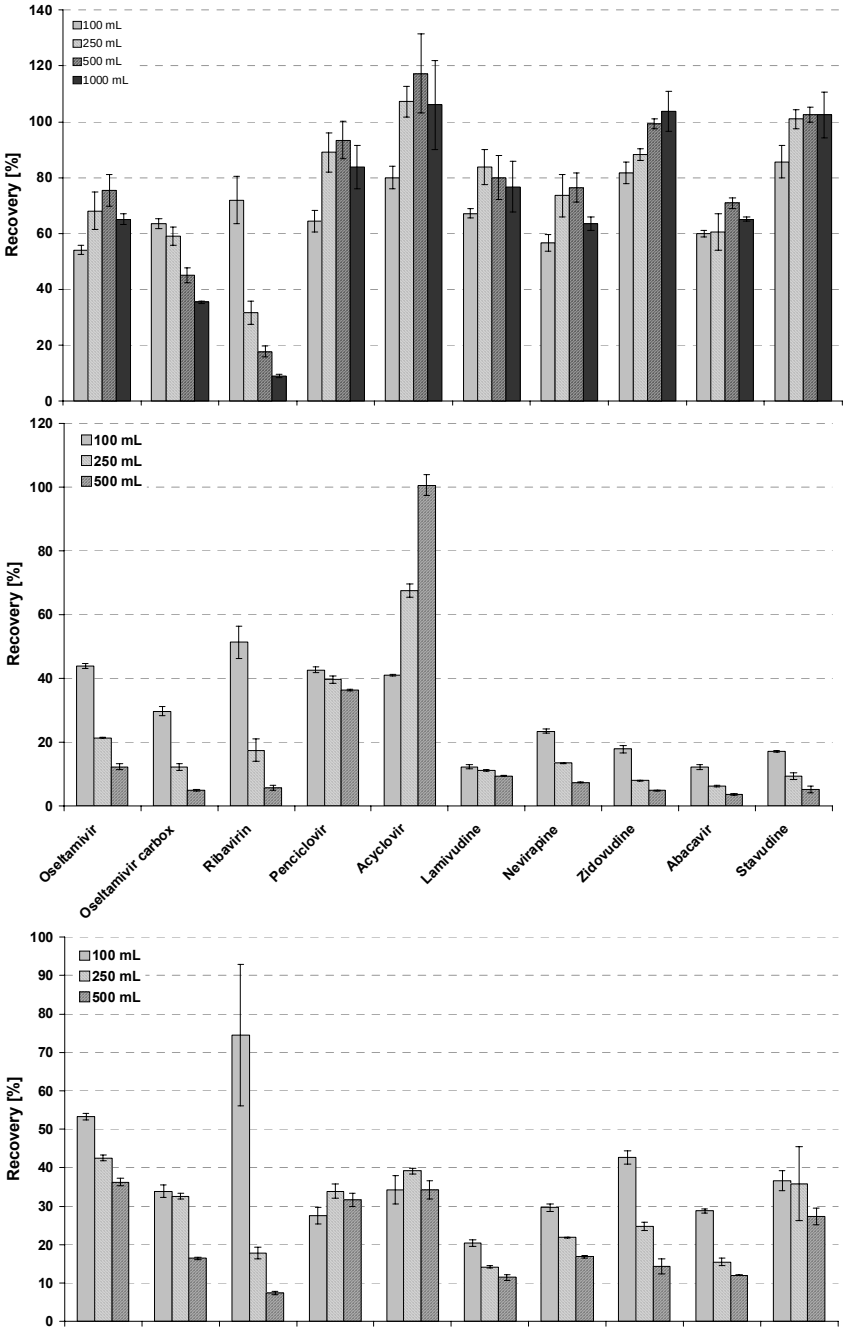

Figure S3. Abs. recoveries of antiviral drugs using different sample volumes of groundwater (top), WWTP influent (middle) and effluent (bottom) on Isolute ENV+ cartridges. Standard deviations are given as error bars (n=3).

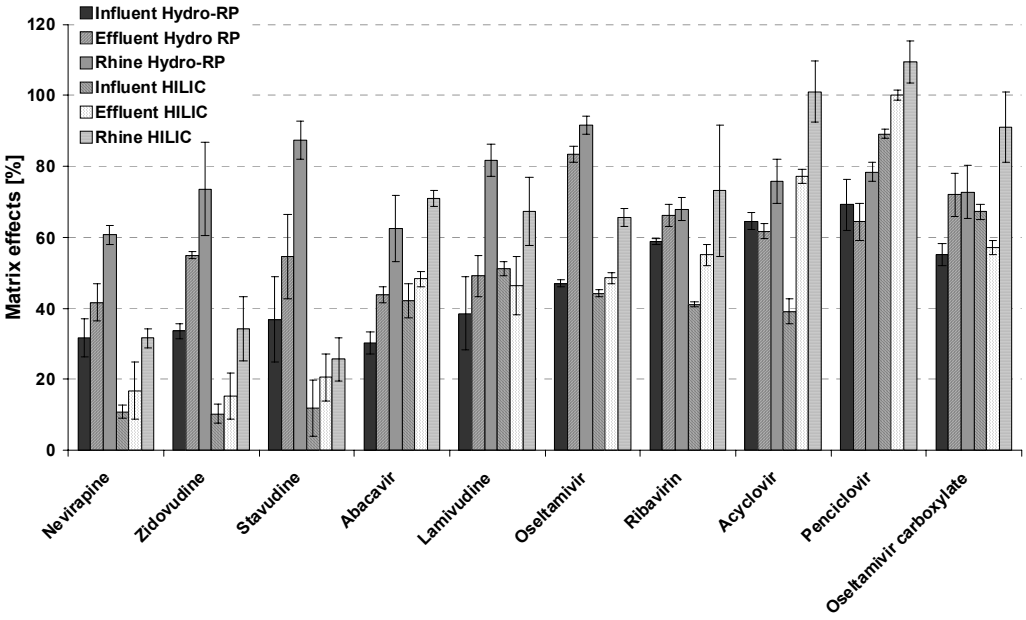

Figure S4. Matrix effects observed in the different investigated matrices depending on the type of chromatographic column used. Analytes are ordered by increasing retention times on the HILIC column. 95 % confidence intervals are given as error bars (n = 3).

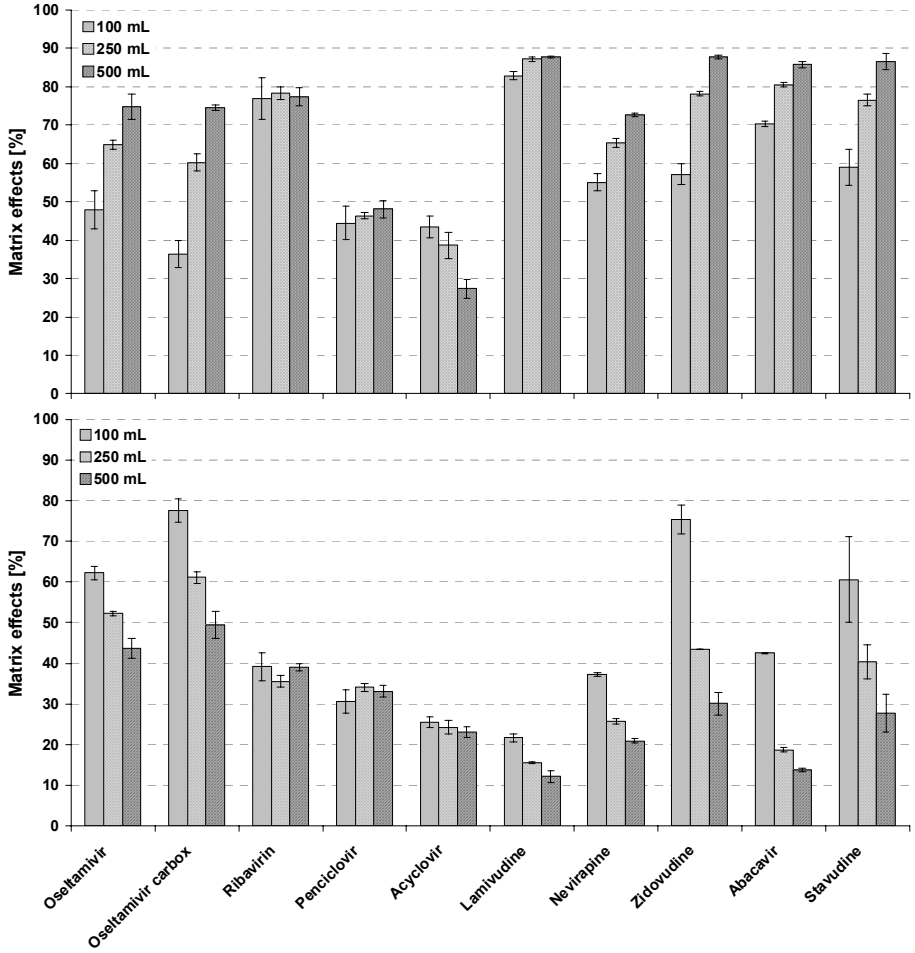

Figure S5. Matrix effects of antiviral drugs using different sample volumes of WWTP influent (top) and WWTP effluent (bottom). Standard deviations are given as error bars (n=3).

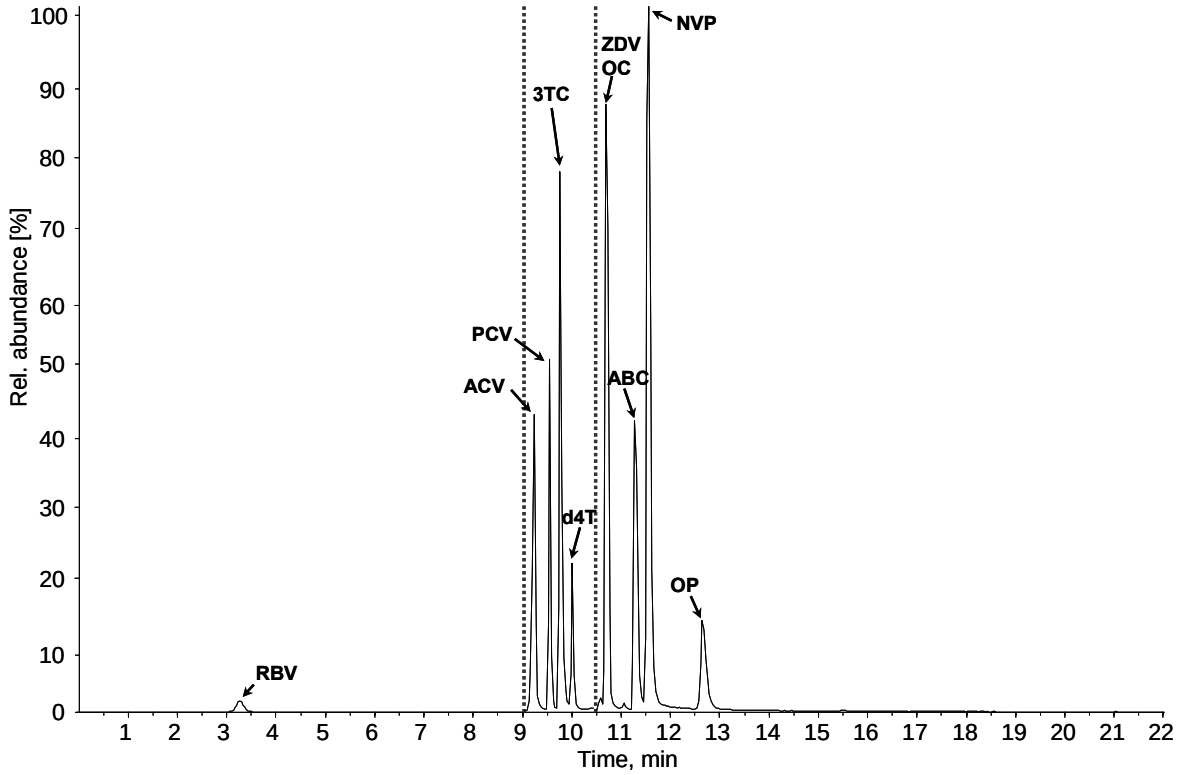

Figure S6. Hydro-RP Total Ion Chromatogram (TIC) obtained for the separation of the target analytes in Rhine River water spiked at a concentration of 100 ng L^{-1}. Dashed vertical lines indicates time windows of used periods. (RBV – ribavirin; ACV – acyclovir; PCV – penciclovir; 3TC – lamivudine; d4t – stavudine; OC – oseltamivir carboxylate; ZDV – zidovudine; ABC – abacavir; NVP – nevirapine; OP – oseltamivir).

References

(1) Friedrich, H.; Mertsch, V., *Statistical database on wastewater treatment in North Rhine Westphalia (NIKLAS)*. Ministry for the Environment and Conservations, Agriculture and Consumer Protection of the state of North Rhine Westphalia: 2004.

(2) Schwabe, U.; Paffrath, D., *Arzneiverordnungs-Report 2008*. Springer: Berlin, Heidelberg, 2008.

(3) Dudley, M. N., Clinical Pharmacokinetics of Nucleoside Antiretroviral Agents. *J. Infect. Dis.* **1995,** *171*, S99-S112.

A.2 SUPPORTING INFORMATION OF CHAPTER 4

Biotransformation of the Antiviral Drugs Acyclovir and Penciclovir in Activated Sludge Treatment

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Content

Pages: 30 Tables: 3 Figures: 21

Tables

Figures

Chemicals and Standards

Acyclovir and penciclovir were purchased from Sigma Aldrich, and labeled standards (acyclovir-d_4 and penciclovir-d_4) were purchased from Campro Scientific (Berlin, Germany). DMSO-d_6 (isotopic enrichment 99.96 %), used as NMR solvent, was purchased from Deutero GmbH (Kastellaun, Germany). Other solvents (*n*-heptane, acetone, methanol and acetonitrile) were picograde and purchased from LGC Promochem (Wesel, Germany). Chemicals were obtained from Carl Roth GmbH (Karlsruhe, Germany) unless noted otherwise.

Informations about sampled WWTPs

WWTP1 serves 330,000 population equivalents (PE) and consists of an aerated grit-removal tank and a primary clarifier followed by biological treatment (nitrification/denitrification). The daily flow rate was approximately 60,000 m³. The activated sludge system is operated with a hydraulic retention time (HRT) and sludge retention time (SRT) of approximately 12 h and 10-12 days, respectively. WWTP2 (230,000 PE; mean daily flow rate: 67,400 m^3 d^{-1}; HRT and SRT of the activated sludge treatment: 12 h and 29 d, respectively) applies the same treatment steps as WWTP1. WWTP3 serves 600,000 PE, has mean daily flow rate of approximately 105,000 m^3, a SRT of 20 d and a HRT of 36 h. Preliminary treatment consists of screens, aerated double grit chambers and primary sedimentation tanks. Biological treatment takes place in individual tanks, with eight tanks operated under anaerobic conditions and 14 tanks operating under anoxic and aerobic conditions, which is followed by secondary sedimentation. Biological phosphate elimination is completed in combination with nitrification and denitrification treatment.

Analysis of acesulfame in aqueous samples

Analysis of acesulfame was performed according to (1). Acesulfame is a heat-resistant artificial sweetener that is used in food, beverages, and pharmaceutical products. Thus, acesulfame will be continuously released from the sewage plants into the river water. Acesulfame was obtained from Sigma Aldrich and acesulfame-d_4 was obtained from Toronto Research Chemicals. Groundwater and river water samples were filtered through glass fiber filters (GF 6, Schleicher and Schuell, Dassel, Germany). Aliquots of 1 mL were spiked with 100 ng of acesulfame-d4. Spiked samples were evaporated to 100µL by a gentle nitrogen stream at 45°C and reconstituted to a volume of 1mL with methanol. The analytes were separated with an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) using a Nucleodur HILIC column, 3mm i.d., 250 mm, 4 µm (Macherrey Nagel, Düren, Germany). An isocratic eluent consisting of 25% ammoniumformiate buffer (25mM) and 75% methanol was used at a flow rate of 0.4 mL/min and 40°C. The injection volume was 25 µL. The compound and source-dependent parameter settings of the LC tandem MS (4000 Q-Trap, Applied Biosystems,

Langen, Germany) are described in Tab 1. Source-dependent parameters are CUR: 25, Temp.: 600°C, GS1: 40, GS2: 40, CAD: high, IS: -4500V, EP: -10V. Calibration curves with 9 calibration points ranging from 0.1 o 1000 µg L^{-1} were prepared by spiking 1mL solution of Milli-Q-water in methanol (1:9; v/v). The linearity was given over the whole calibration range. Correlation coefficient was 0.9998. Limit of quantification (LOQ) was set to the lowest calibration point. Signal/noise ratios of the analytes at LOQ were set to be higher than 50.

TP fractionation via HPLC/UV

To fractionate the TPs, a Waters HPLC system was used, equipped with a Waters 717 plus autosampler, column oven, Waters 600 controller with quaternary pump, in-line degasser and Waters 2487 dual-wavelength absorbance detector (operated at 254 and 280 nm). Isolation of individual TPs was achieved by chromatographic separation on a semi-preparative Hydro-RP column (250 x 10 mm; 4 µm; Phenomenex, Aschaffenburg, Germany), in which aliquots of 500 µL were injected into the column. Individual fractions were collected with an automated sample collector (Advantec SF-2120 super fraction collector, Techlab GmbH, Erkerode, Germany). As mobile phase, 0.2 % formic acid (A) and methanol + 0.1 % formic acid (B) were used. An eluent gradient was applied to achieve separation of the analytes. The percentage of (A) was changed linearly as follows: 0-15 min, 90%; 30 min, 30%; 33 min, 30%; 36 min, 90%. The total run time was 50 min. The flow rate was set to 2 mL min^{-1} and the column oven was operated at 40°C.

HR-MS and HR-MS/MS analysis of TPs via LTQ Orbitrap Velos ESI FT-MS

Chromatographic conditions were the same as for quantification by LC-tandem MS (see below). The ESI source parameters were set as follows: capillary temperature: 275 °C; capillary voltage 3.0 kV; heater temperature 300 °C; sheath gas flow rate 30 arbitrary units (AU); aux gas flow rate 10 AU; S-lens RF level 69%. Data dependent acquisition was used to conduct MS^2 and MS^3 spectra as follows: a full scan (100 – 500 m/z; positive mode) was performed followed by MS^2 and MS^3 scans for the 2 most intense ions with an intensity of >10000 counts per second (cps) and >1000 cps, respectively. CID (collision induced dissociation) with a normalized collision energy of 35% was used for fragmentation with an activation time of 10 ms. In addition, dynamic exclusion was applied (exclusion of masses for which 3 MS^n experiments have been performed; exclusion duration: 30 s) enabling also MS^n experiments for less abundant ions (e.g. during co-elution of different substances).

NMR analysis

The structures of the transformation products were investigated on a Bruker Avance III 500 (11,7 T) with a 5 mm z-gradient BBFO ^{1}H/X probe. The proton and carbon spectra were measured in DMSO-d_6 at 298,3K and the spectra were referenced as follows: for the residual DMSO-(H)-d_5 δ(^{1}H) = 2,49 ppm and DMSO-d_6 δ(^{13}C) = 39,5 ppm.

A standard ^{1}H NMR spectrum needed between 32 and 128 transients attained with a 11 µs 90° pulse, 10000 Hz spectral width and a recycling delay between 1 and 10 s. ^{13}C NMR (125 MHz) experiments were obtained with ^{1}H decoupled (powergate decoupling using 30° degree flip angle) ^{13}C NMR acquisition methods and a spectral width of 25000 Hz.

The assignment was accomplished by ^{1}H,^{1}H COSY 2D method. The spectroscopic widths of the homo-nuclear 2D COSY experiments were typically 14000 Hz in both dimension (f1 and f2) and the relaxation delay 1,2s. The 2D ^{1}H,^{13}C-heteronuclear single quantum correlations (HSQC) were registered at a Bruker Avance III 500 system and Avance III 700. A 5 mm z-gradient TBI ^{1}H/^{31}P/BB probe was used on the 700 MHz system. The spectroscopic width for the ^{1}H window was 10000 Hz (500MHz) or 14000 Hz (700 MHz) and for the ^{13}C 25000 Hz (125 MHz) or 35000 Hz (176 MHz).

All the 2D ^{1}H,^{13}C-HSQC experiments were run by using 8192 points in f2 and 512 points in f1. Before Fourier transformation, the data were zero filled to 1024 points in f1 and multiplied by a window function (q-sine bell) in both dimension.

The following parameters were used to obtain optimal results: $^{1}J_{CH}$=145 Hz for optimizing observable intensities of cross peaks from one bond ^{1}H-^{13}C correlation, a relaxation delay of 2 s, while typical π/2 pulse width for ^{1}H were 11 µs (500 MHz) or 10,8 µs and ^{13}C and 9,5 µs (125 MHz) or 17 µs (176 MHz system).

Analysis of aqueous environmental samples via SPE and LC-tandem MS detection

Solid Phase Extraction (SPE).

For SPE 100 mL of raw wastewater, 200 mL of treated wastewater and 500 mL of surface water and drinking water were spiked with 50 ng of surrogate standards (acyclovir-d_4 and penciclovir-d_4). Prior to SPE, water samples were filtered (< 1 µm) and adjusted to pH 2.5 and pH 4 for PCV TPs and to pH 2.5 for the analysis of Carboxy-ACV using 3.5 M H_2SO_4. Isolute ENV+ cartridges (6 mL, 500 mg; Biotage, Uppsala, Sweden) were conditioned using 1 x 2 mL *n*-heptane, 1 x 2 mL acetone, 3 x 2 mL methanol followed by 4 x 2 mL groundwater (pH 2.5 or pH 4). Samples were passed through the cartridges with a flow rate of approx. 10 mL min^{-1}. Prior to the elution of the analytes using 5 x 2 mL acetone/methanol (70/30, v/v), cartridges were dried using a gentle stream of nitrogen. Sample extracts were evaporated to 50 µL using a gentle stream of nitrogen and were reconstituted to 1 mL with 0.1 % formic acid.

Liquid Chromatography-Tandem Mass Spectrometry.

LC–ESI-tandem MS was run in positive mode for the detection of ACV, PCV as well as their TPs formed in environmental samples. Detailed information on the setup and the ESI parameters applied can be found in Prasse et al. (2010). A Hydro-RP column (150 x 3 mm; Phenomenex, Aschaffenburg, Germany) was used for chromatographic separation using 0.2% formic acid (A) and methanol + 0.1% formic acid (B) as mobile phases. The gradient of (A) was as follows: 0-4 min, 100%; 7 min, 30%; 17 min, 10%; 18 min, 100%. Total run time was 22 min. The column oven was set to 40°C, flow rate to 0.4 mL min^{-1} and injection volume was 10 µL. Optimization of MS/MS parameters for individual TPs was performed in continuous flow mode, injecting 1 µg mL^{-1} standard solutions at a flow rate of 10 µL min^{-1} with a syringe pump (Table S1).

Method validation.

For determining the absolute recoveries of the analytes, peak areas in spiked samples (groundwater, surface water, WWTP influent and effluent) were compared with the peak areas in an external standard containing the same amount of analytes (for details see Prasse et al. (*2*). Relative recoveries were calculated by dividing the measured quantities with the nominal (spiked) quantities. Four replicates were used. LOQ values were defined as the lowest calibration point in the linear regression with a S/N of at least 10 and 3 for MRM1 and MRM2, respectively.

Quantification.

Due to the absence of PCV TPs in all investigated samples, quantification was only performed for ACV and Carboxy-ACV. Therefore, a matrix-matched calibration ranging from 1 to 2000 ng L^{-1} (7 points) was prepared by spiking respective quantities of both analytes into 500 mL of groundwater. A constant quantity of surrogate standard (acyclovir-d_4, 50 ng) was added. Samples were extracted using SPE as described above. Linear regression was applied to the calibration curves with a weighing factor of 1/x.

Table S1. Precursor, product ions and MS parameters used for LC/MS/MS detection[a]

Substance	MRM 1	MRM 2	DP [V]	CE (MRM 1 / MRM 2) [eV]	CXP (MRM 1 / MRM 2) [V]
Analytes					
Acyclovir (ACV)	226.0 → 152.0	226.0 → 135.0	71	17 / 43	12 / 14
Carboxy-ACV	240.0 → 152.0	240.0 → 135.0	46	19 / 43	12 / 12
Penciclovir (PCV)	254.1→ 152.0	254.1 → 135.0	91	25 / 45	10 / 12
PCV TP209	210.0 → 152.0	210.0 → 135.1	76	29 / 43	12 / 10
PCV TP281	282.0 → 238.2	282.0 → 152.0	66	19 / 29	20 / 12
PCV TP249A	250.0 → 152.2	250.0 → 135.1	61	23 / 45	12 / 10
PCV TP249B	250.0 → 152.1	250.0 → 99.0	61	25 / 29	12 / 6
PCV TP251	252.0 → 152.0	250.0 → 135.1	66	23 / 44	12 / 10
PCV TP267	268.0 → 152.0	268.0 → 135.1	61	27 / 51	12 / 10
PCV TP237	238.1 → 152.0	238.1 → 135.0	66	25 / 41	28 / 8
PCV TP233	234.0 → 83.1	234.0 → 216.0	61	25 / 25	6 / 18
Internal Standards					
Acyclovir-d_4	230.3 → 152.0	230.3 → 135.1	46	19 / 41	12 / 10
Penciclovir-d_4	258.3 → 152.1	258.3 → 135.1	66	27 / 49	0 / 8

[a] DP = Declustering potential; CE = Collision energy; CXP = Collision cell exit potential

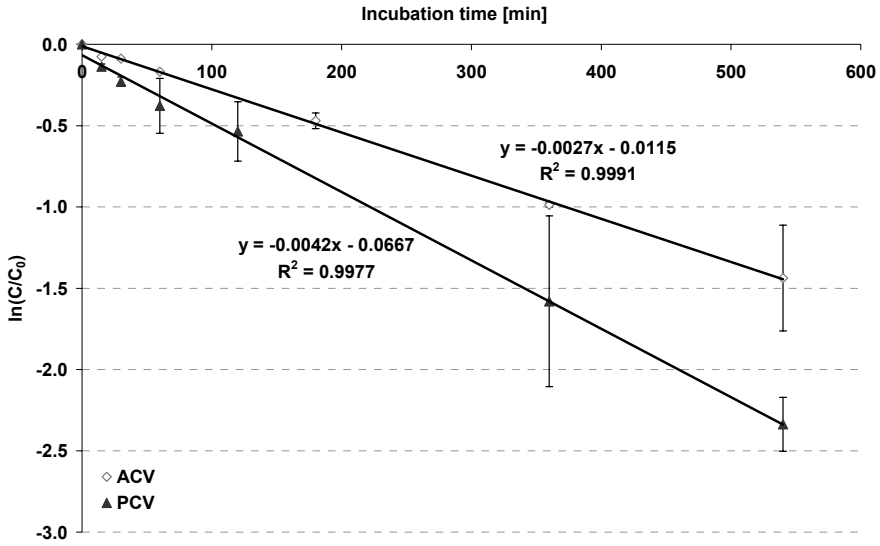


Figure S7. Degradation of ACV and PCV in sewage sludge (0.4 g_{ss} L^{-1}) at a concentration of 4 µg L^{-1}. Linear regressions are given assuming a first order kinetic.

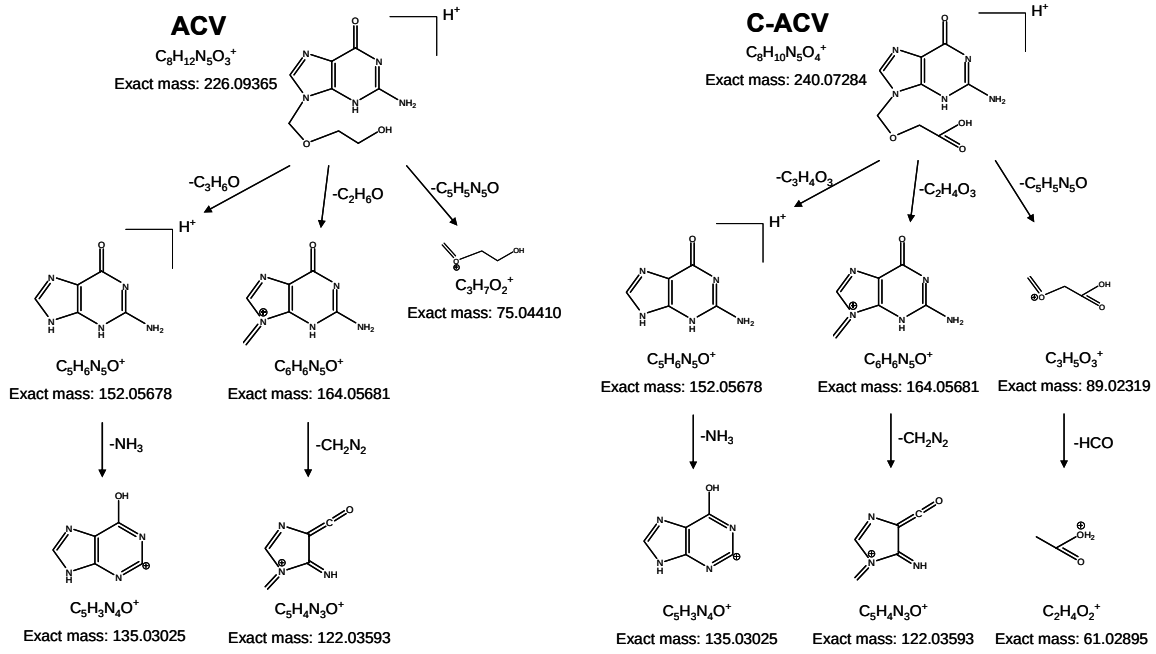
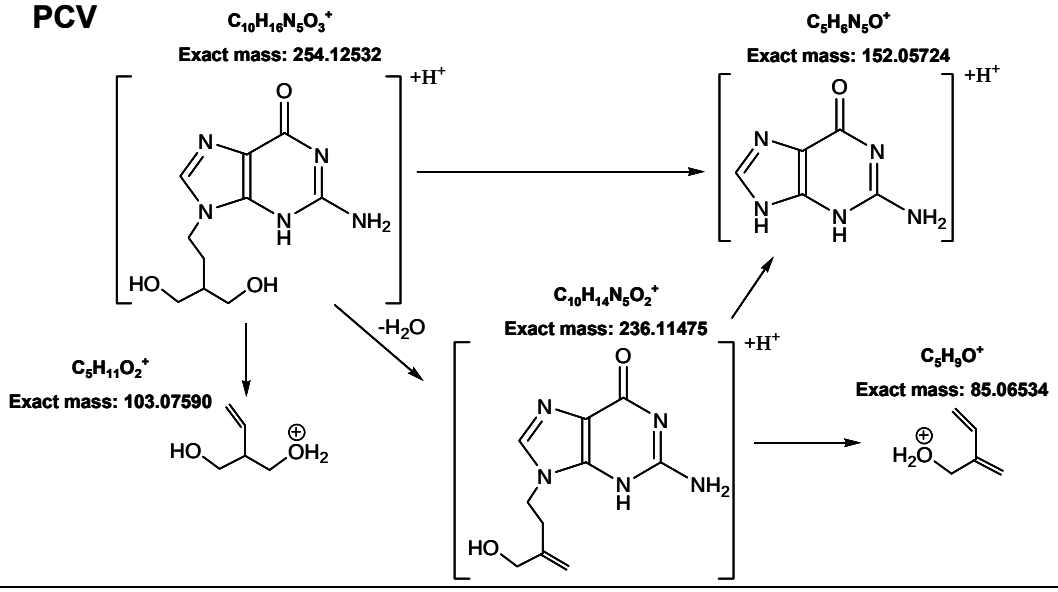
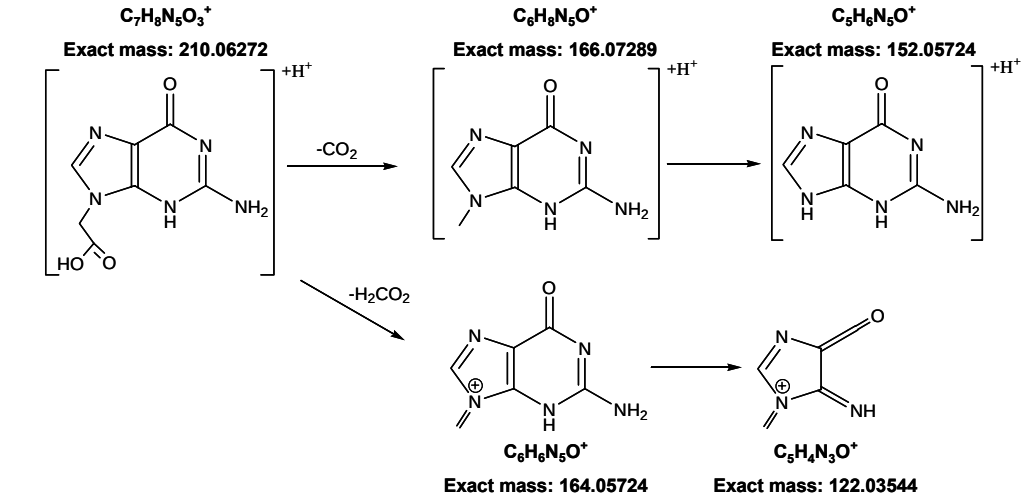


Figure S8. Proposed fragmentation pathway of ACV (left) and Carboxy-ACV (right).





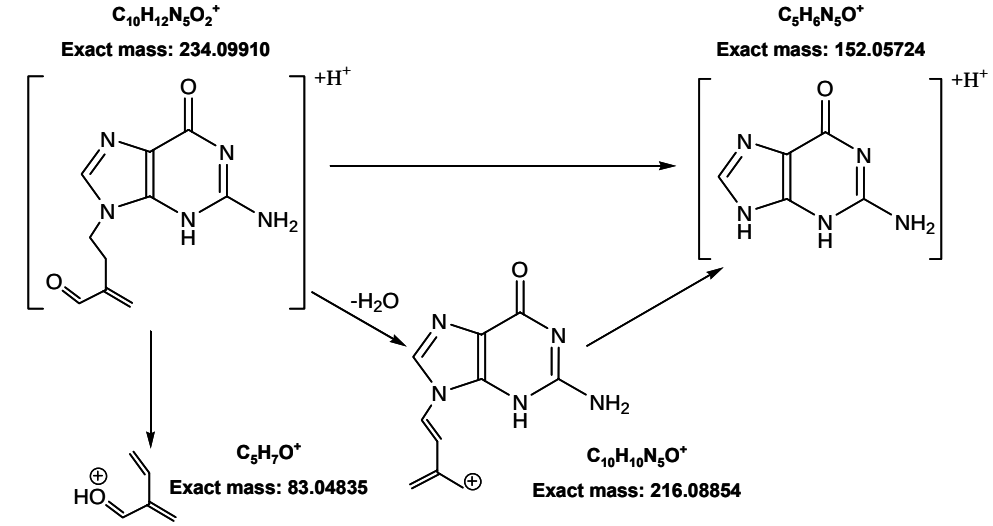
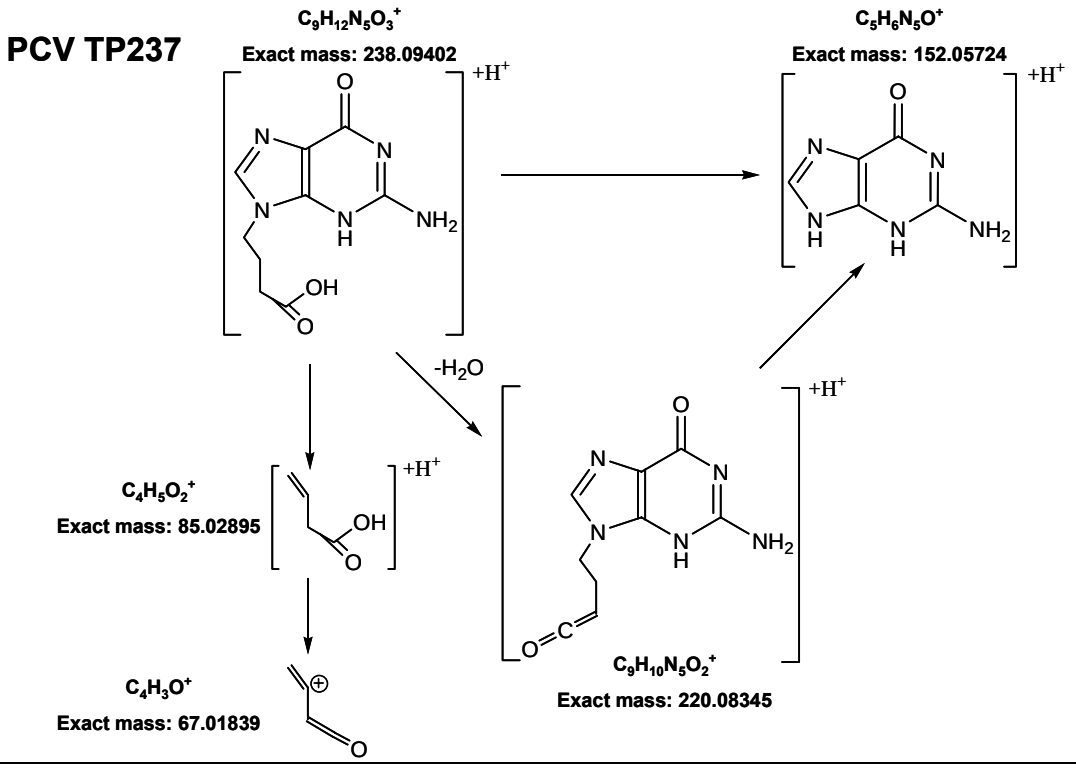


Figure S9. Proposed fragmentation pathway of PCV, PCV TP209 and TP233.



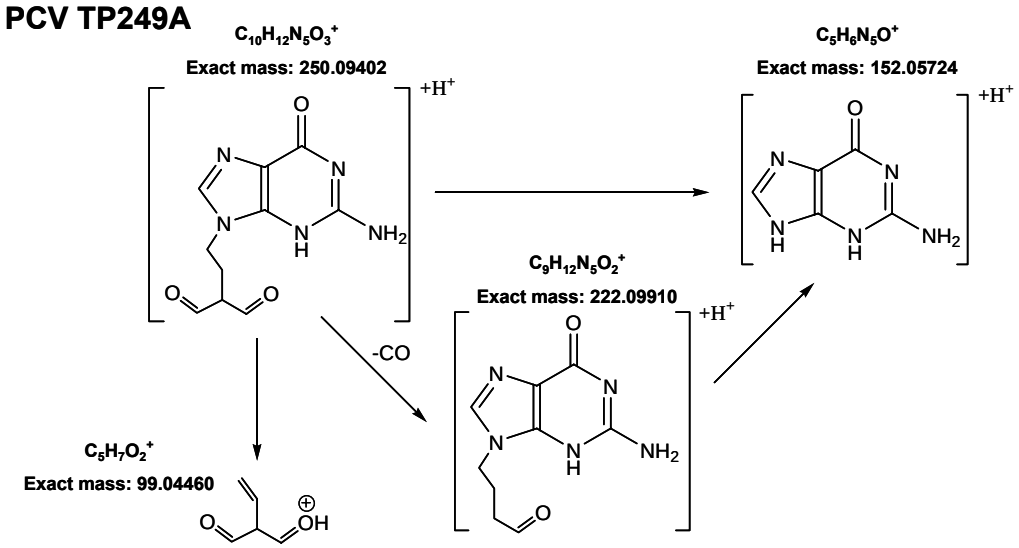
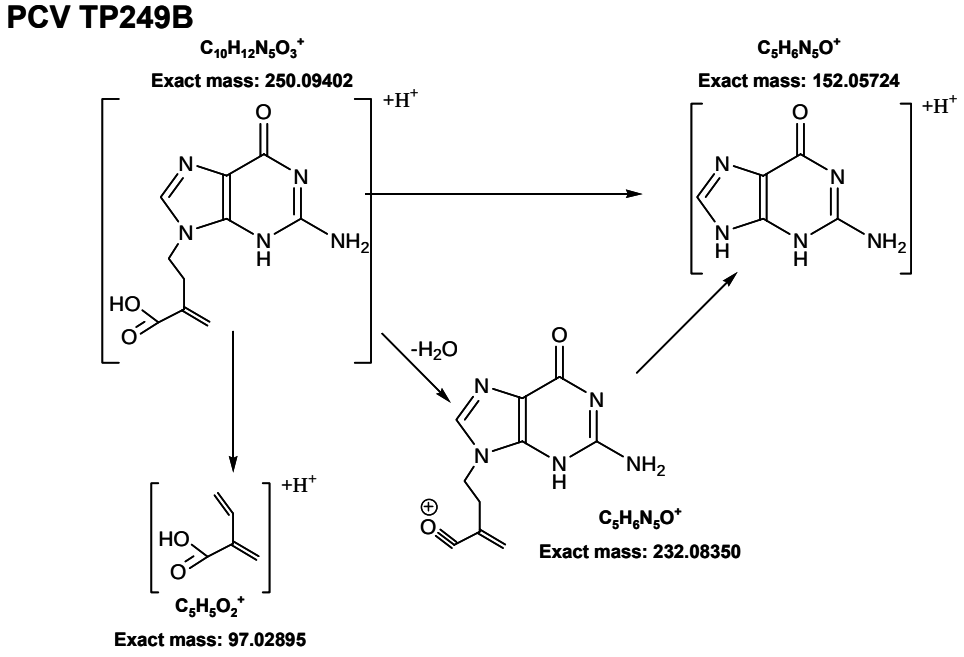


Figure S10. Proposed fragmentation pathway of PCV TP237 and TP249A.



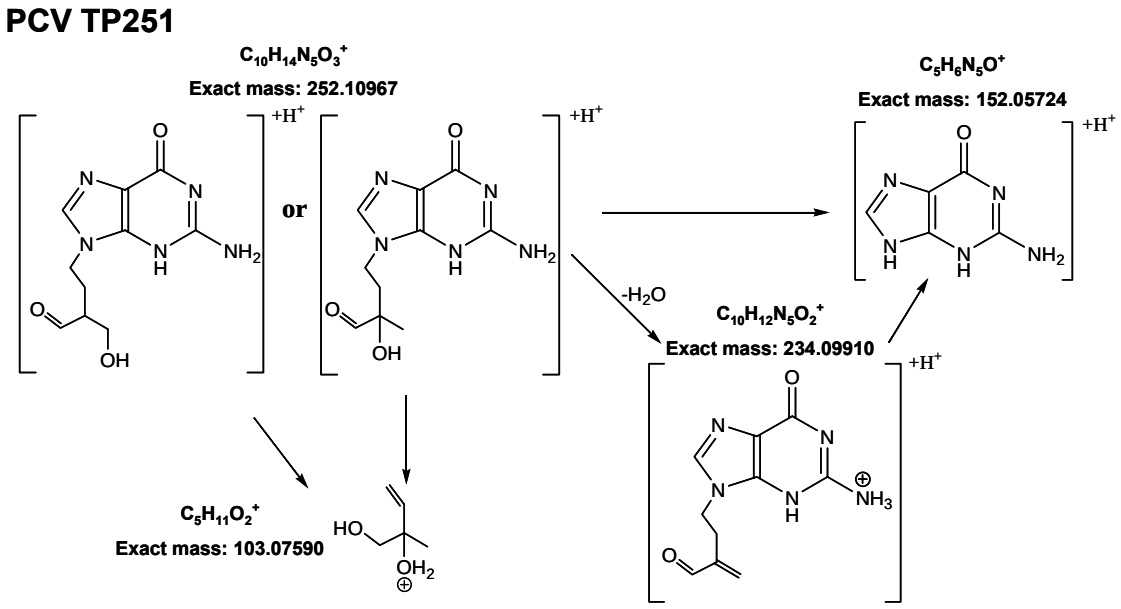


Figure S11. Proposed fragmentation pathway of PCV TP249B and TP251.

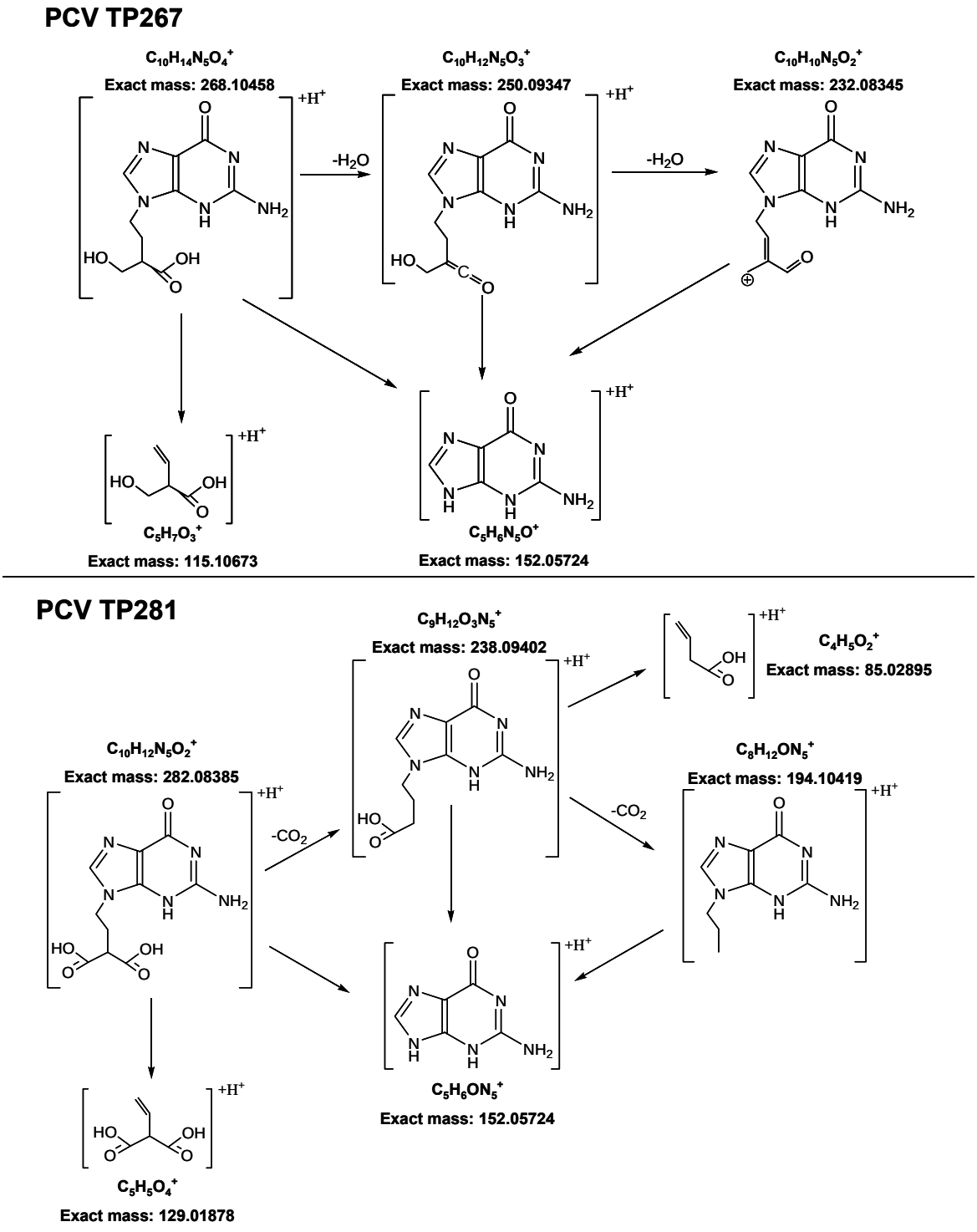


Figure S12. Proposed fragmentation pathway of PCV TP267 and TP281.

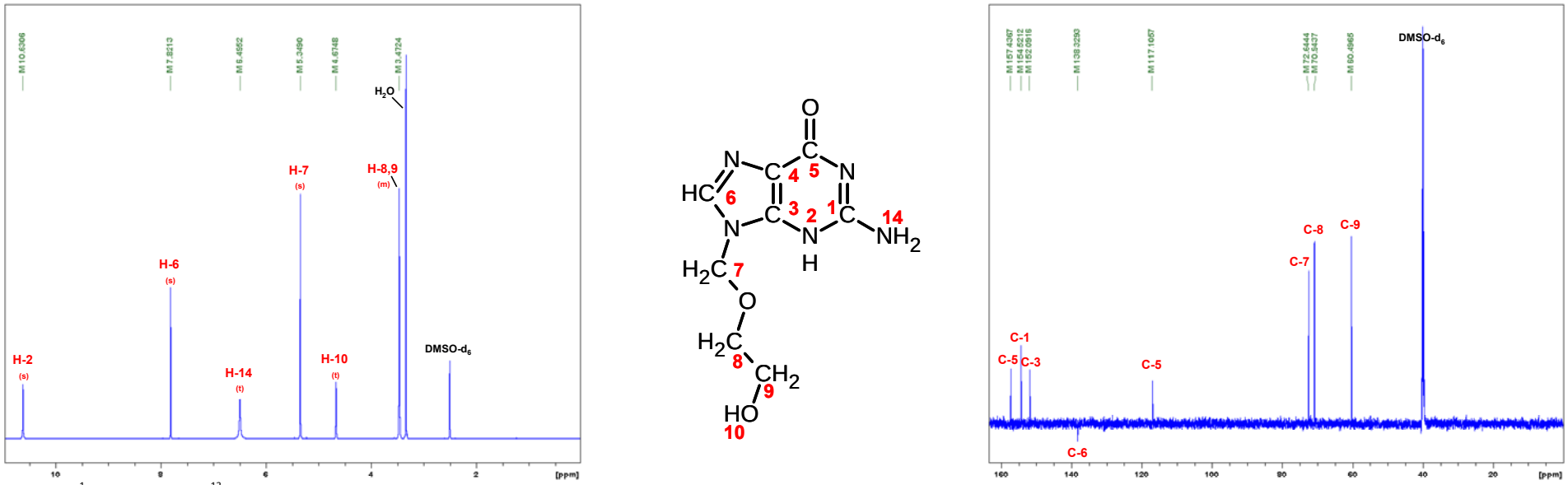
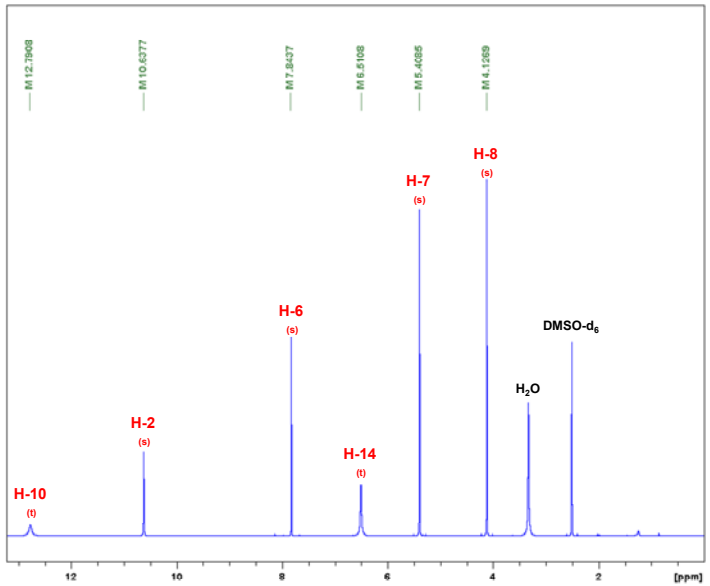
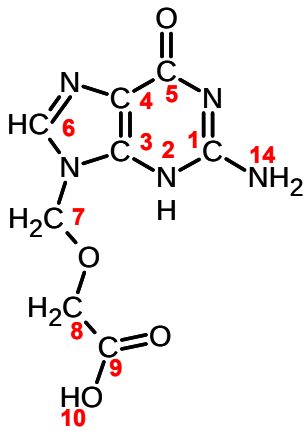


Figure S13. ^{1}H-NMR (left) and ^{13}C-NMR (right) spectra of ACV. All samples were measured at 298.3 K in DMSO-d_6.





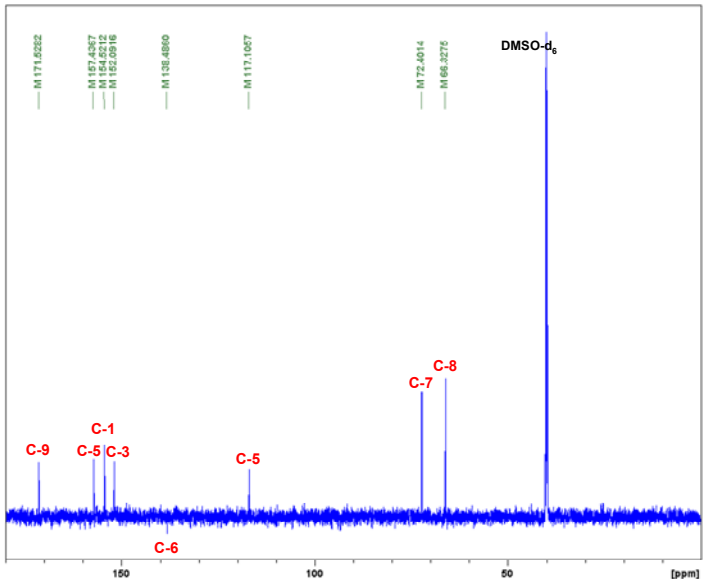


Figure S14. ^{1}H-NMR (left) and ^{13}C-NMR (right) spectra of Carboxy-ACV. All samples were measured at 298.3 K in DMSO-d_6.

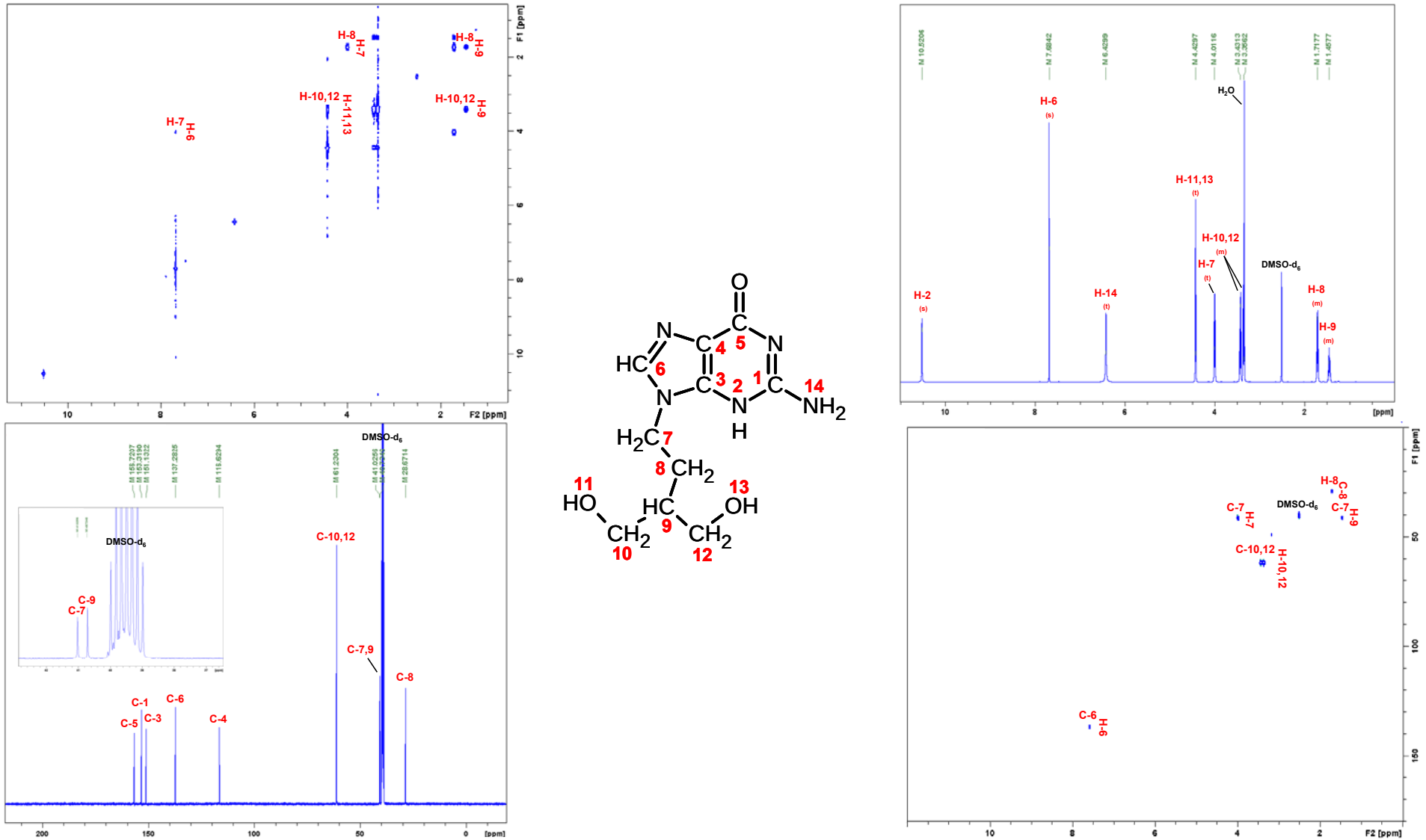


Figure S15. ^{1}H,^{1}H-COSY (top left), ^{1}H-NMR (top right), ^{13}C-NMR (bottom left) and ^{1}H,^{13}C-HSQC (bottom right) spectra of PCV. All samples were measured at 298.3 K in DMSO-d_6.

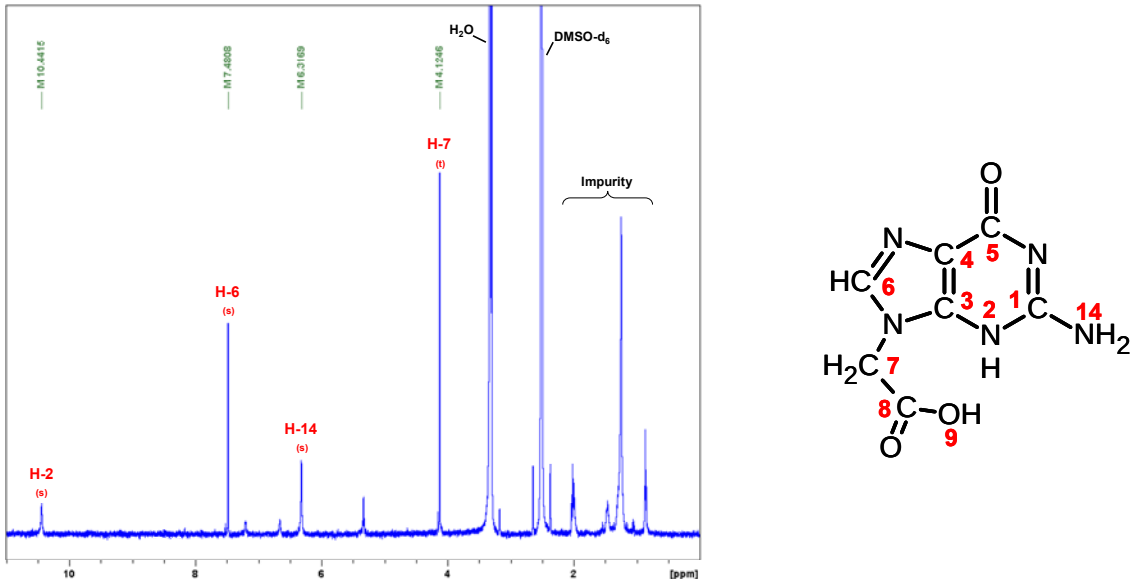


Figure S16. ^{1}H-NMR (left) and ^{1}H,^{13}C-HSQC (right) spectra of PCV TP209. All samples were measured at 298.3 K in DMSO-d_6.

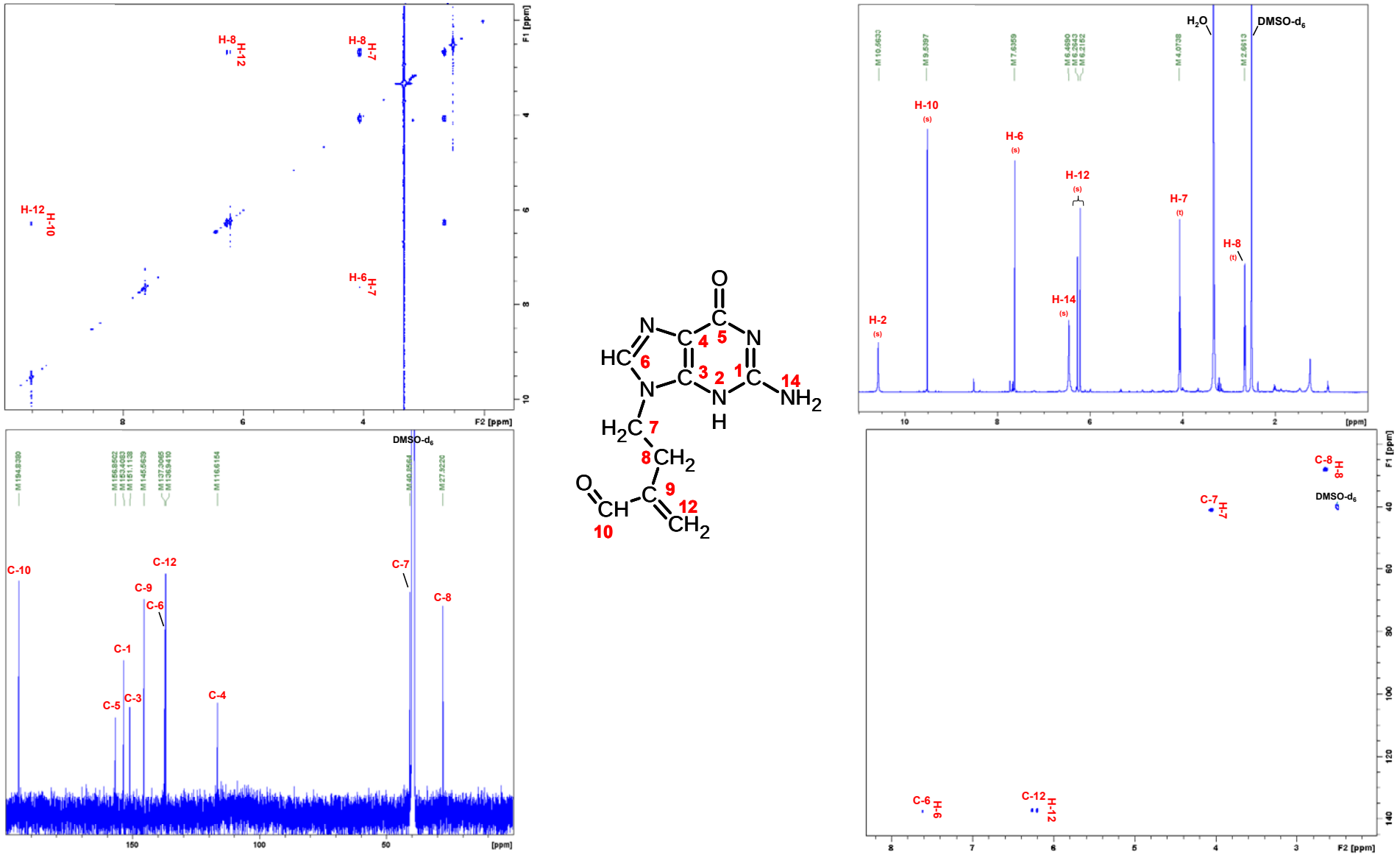


Figure S17. ^{1}H,^{1}H-COSY (top left), ^{1}H-NMR (top right), ^{13}C-NMR (bottom left) and ^{1}H,^{13}C-HSQC (bottom right) spectra of PCV TP233. All samples were measured at 298.3 K in DMSO-d_6.

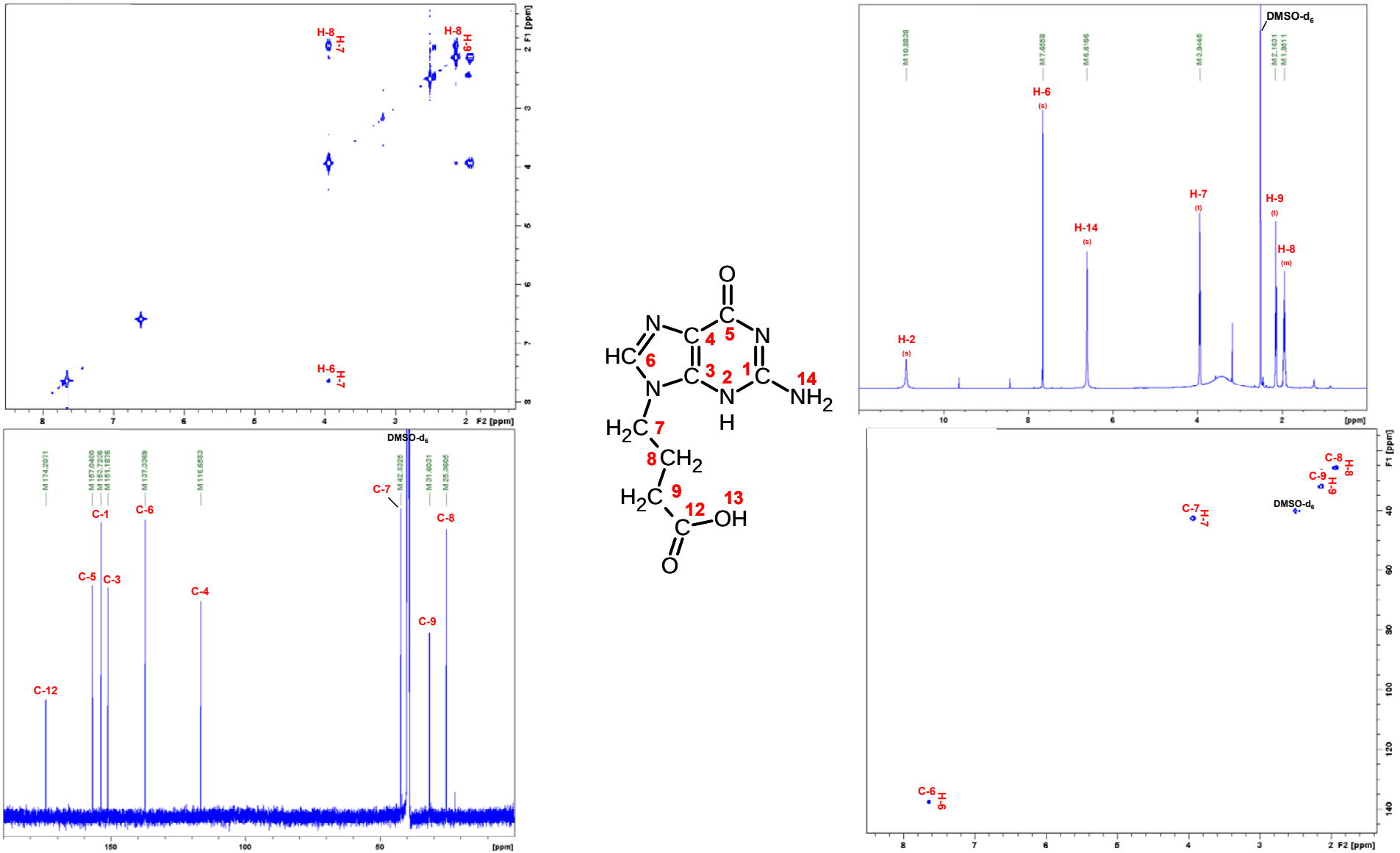


Figure S18. ^{1}H,^{1}H-COSY (top left), ^{1}H-NMR (top right), ^{13}C-NMR (bottom left) and ^{1}H,^{13}C-HSQC (bottom right) spectra of PCV TP237. All samples were measured at 298.3 K in DMSO-d_6.

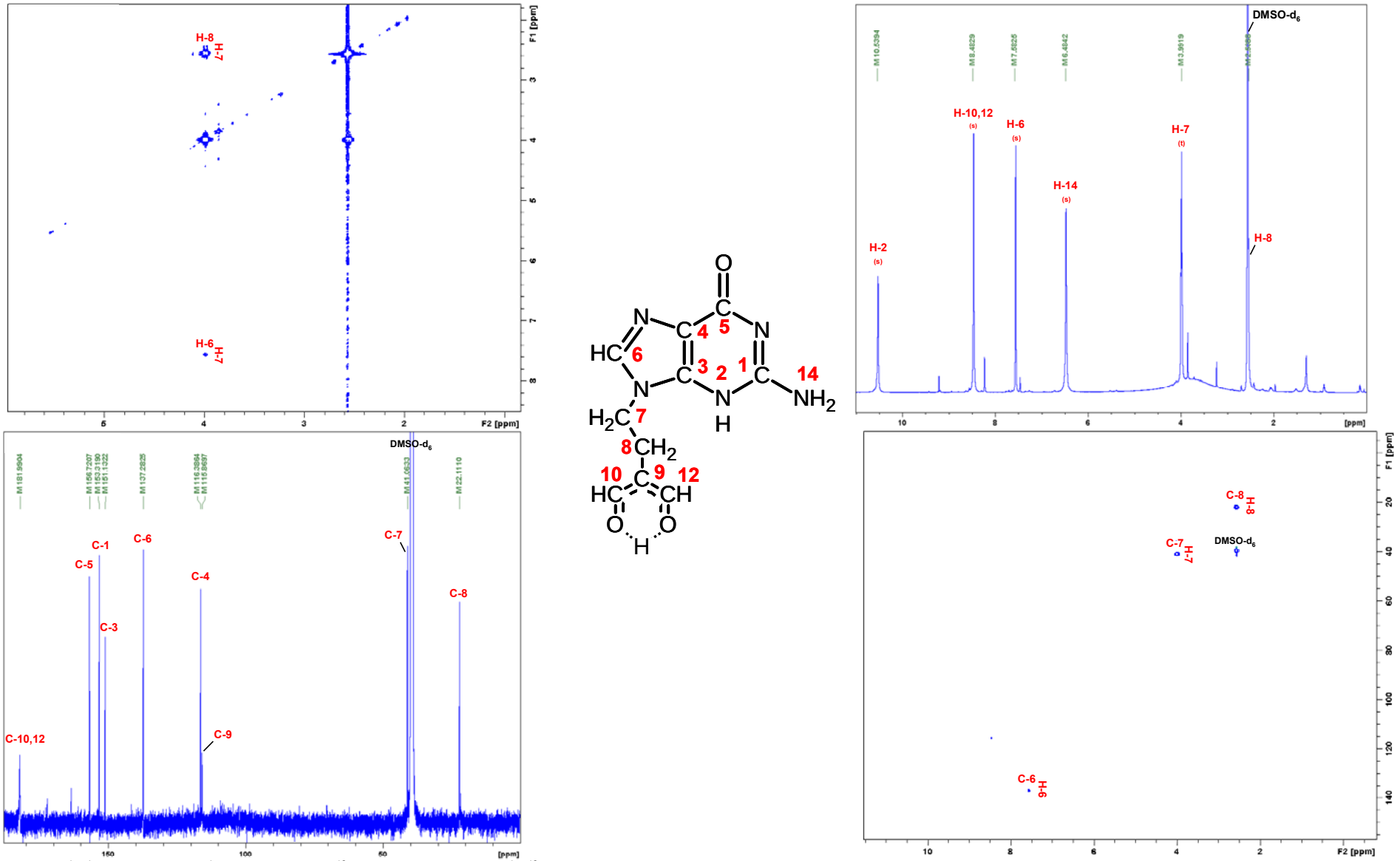


Figure S19. ^{1}H,^{1}H-COSY (top left), ^{1}H-NMR (top right), ^{13}C-NMR (bottom left) and ^{1}H,^{13}C-HSQC (bottom right) spectra of PCV TP249A. All samples were measured at 298.3 K in DMSO-d_6.

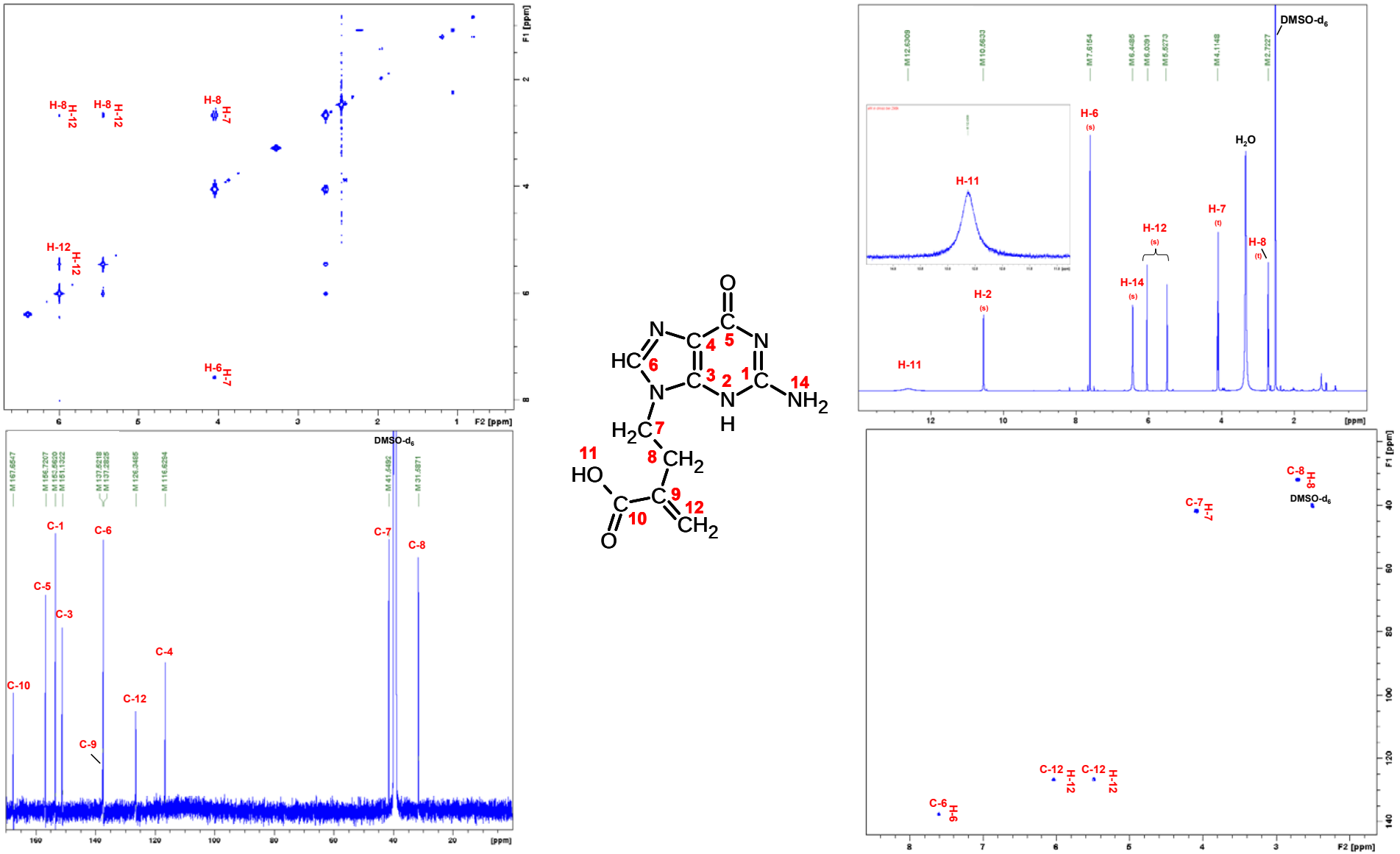


Figure S20. ^{1}H,^{1}H-COSY (top left), ^{1}H-NMR (top right), ^{13}C-NMR (bottom left) and ^{1}H,^{13}C-HSQC (bottom right) spectra of PCV TP259B. All samples were measured at 298.3 K in DMSO-d_6.

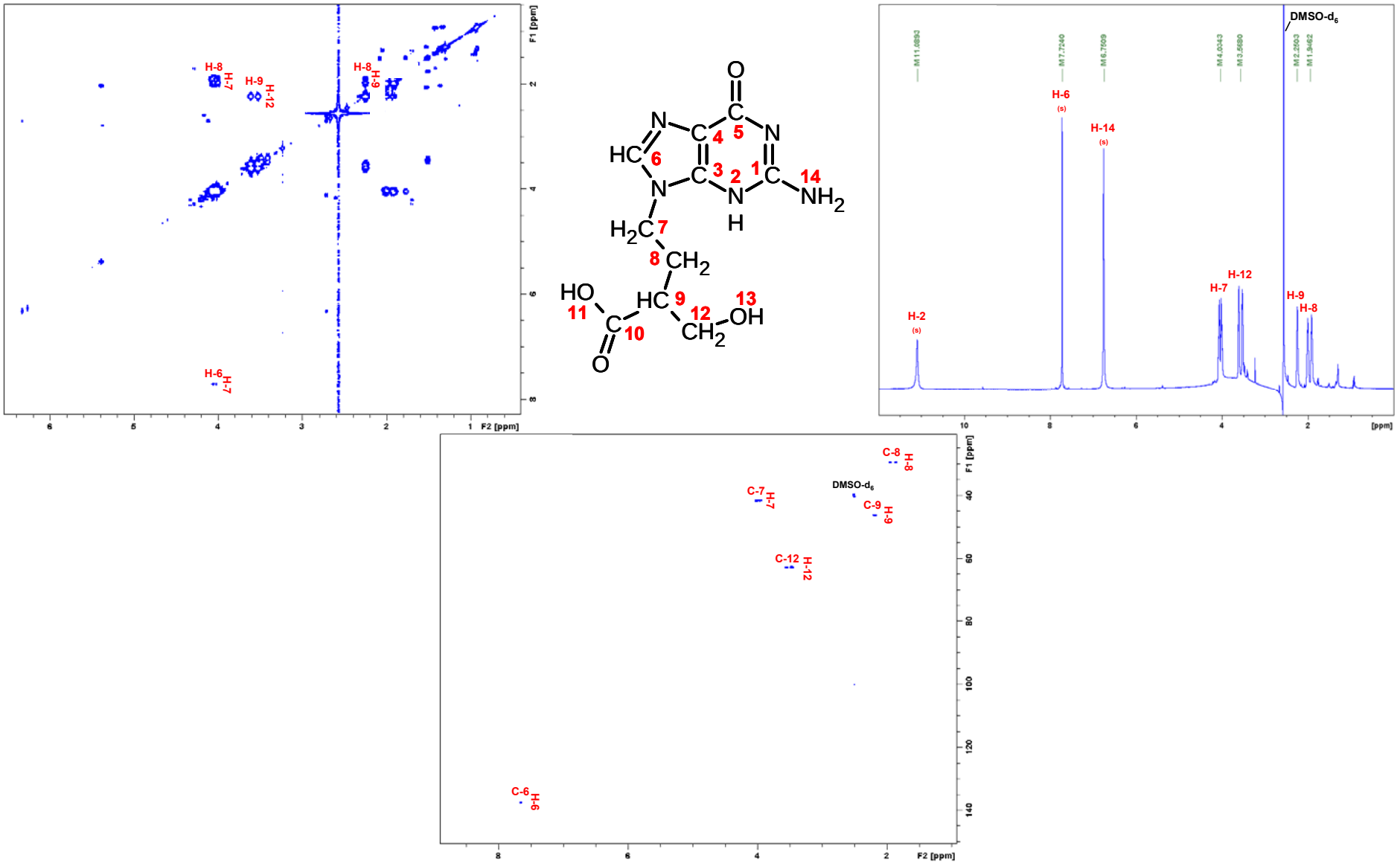


Figure S21. ^{1}H,^{1}H-COSY (top left), ^{1}H-NMR (top right) and ^{1}H,^{13}C-HSQC (bottom) spectra of PCV TP267. All samples were measured at 298.3 K in DMSO-d_6.

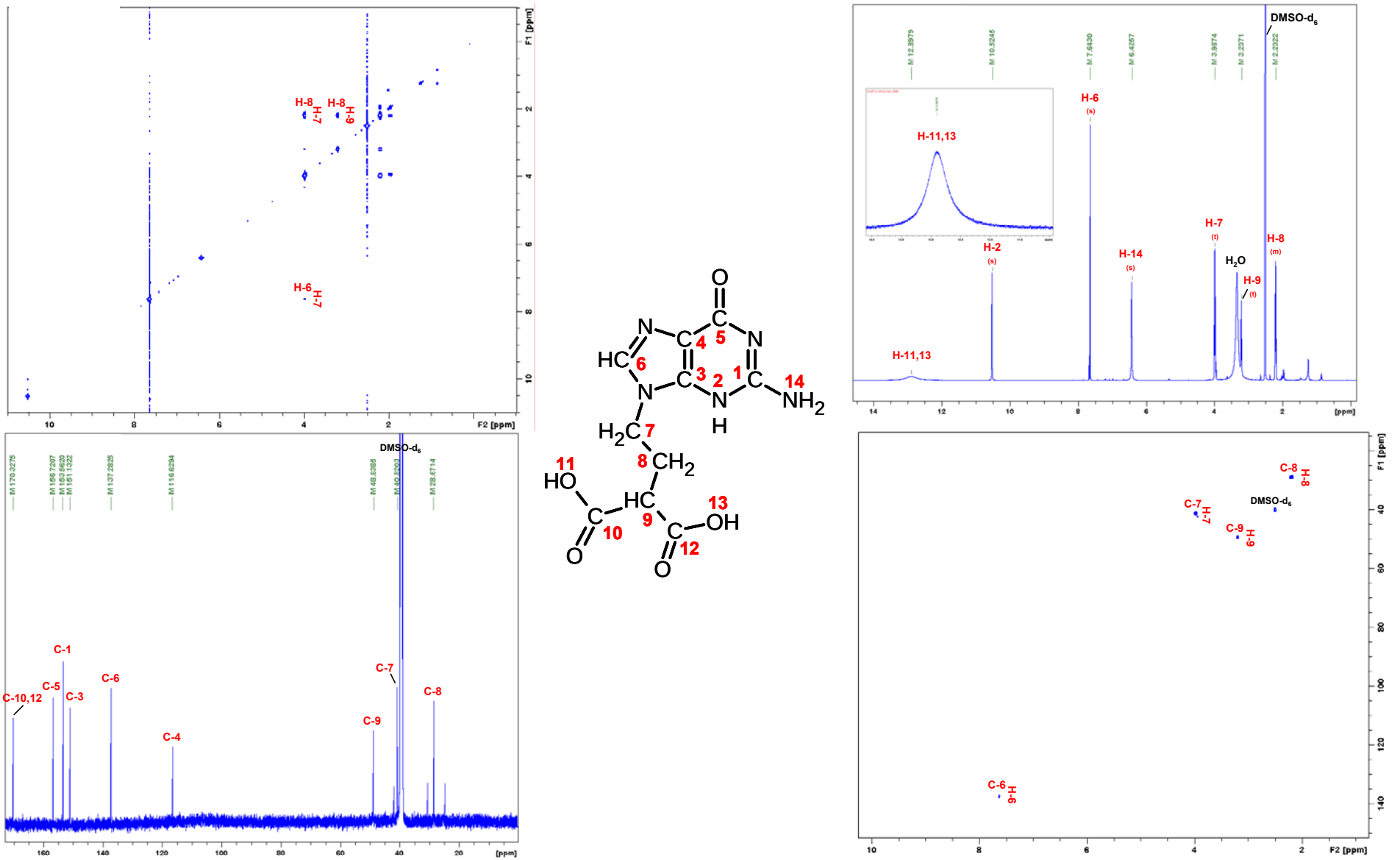


Figure S22. ^{1}H,^{1}H-COSY (top left), ^{1}H-NMR (top right), ^{13}C-NMR (bottom left) and ^{1}H,^{13}C-HSQC (bottom right) spectra of PCV TP281. All samples were measured at 298.3 K in DMSO-*d*$_6$.

Table S2. Absolute and relative recoveries together with LOQ values of ACV and Carboxy-ACV in the investigated matrices using ENV+ SPE-cartridges at pH 2.5 (n=4).

	Groundwater				
Compound	abs. recovery [%] 50 ng	rel. recovery [%] 50 ng	abs. recovery [%] 500 ng	rel. recovery [%] 500 ng	LOQ [ng L^{-1}]
ACV	*n.d.*	*n.d.*	110 ± 4	96 ± 5	1.0
Carboxy-ACV	54 ± 9	90 ± 10	80 ± 5	93 ± 8	1.0

	Surface water				
Compound	abs. recovery [%] 50 ng	rel. recovery [%] 50 ng	abs. recovery [%] 500 ng	rel. recovery [%] 500 ng	LOQ [ng L^{-1}]
ACV	*n.d.*	*n.d.*	107 ± 7	93 ± 5	1.0
Carboxy-ACV	96 ± 3	105 ± 3	88 ± 1	102 ± 3	1.0

	WWTP influent		
Compound	abs. recovery [%] 500 ng	rel. recovery [%] 500 ng	LOQ [ng L^{-1}]
ACV	96 ± 9	86 ± 6	5.0
Carboxy-ACV	85 ± 4	108 ± 3	5.0

	WWTP effluent		
Compound	abs. recovery [%] 500 ng	rel. recovery [%] 500 ng	LOQ [ng L^{-1}]
ACV	94 ±6	93 ± 4	2.5
Carboxy-ACV	81 ± 7	113 ± 18	2.5

n.d. not determined

Table S3. Absolute recoveries (mean ± 95% confidence interval) and LOQ values of the identified PCV TPs in investigated aqueous matrices (n=4; 50 ng spiked). Optimal pH for individual PCV TPs is highlighted in grey.

	[%]							
pH 2.5	**PCV TP267**	**PCV TP237**	**PCV TP249A**	**PCV TP249B**	**PCV TP281**	**PCV TP209**	**PCV TP233**	**PCV TP251**
LOQ [ng L^{-1}] groundwater	30	30	100	8	100	200	150	150
Groundwater	71 ± 6	64 ± 16	62 ± 13	100 ± 5	71 ± 3	57 ± 11	41 ± 11	86 ± 12
Surface water	104 ± 6	85 ± 9	56 ± 13	88 ± 5	104 ± 10	84 ± 4	55 ± 13	82 ± 4
WWTP effluent	84 ± 9	85 ± 8	41 ± 5	75 ± 8	85 ± 16	84 ± 19	83 ± 25	86 ± 10
WWTP influent	85 ± 7	83 ± 15	43 ± 12	73 ± 4	94 ± 5	73 ± 10	59 ± 21	77 ± 12
pH 4.0								
Groundwater	71 ± 4	102 ± 1	63 ± 11	100 ± 10	2 ± 2	6 ± 2	97 ± 21	83 ± 8
Surface water	86 ± 8	97 ± 5	75 ± 10	101 ± 5	10 ± 8	4 ± 3	62 ± 21	91 ± 11
WWTP effluent	100 ± 12	101 ± 6	97 ± 24	89 ± 13	35 ± 6	8 ± 6	114 ± 8	95 ± 15
WWTP influent	104 ± 9	102 ± 2	87 ± 14	77 ± 2	53 ± 12	25 ± 6	68 ± 21	79 ± 13

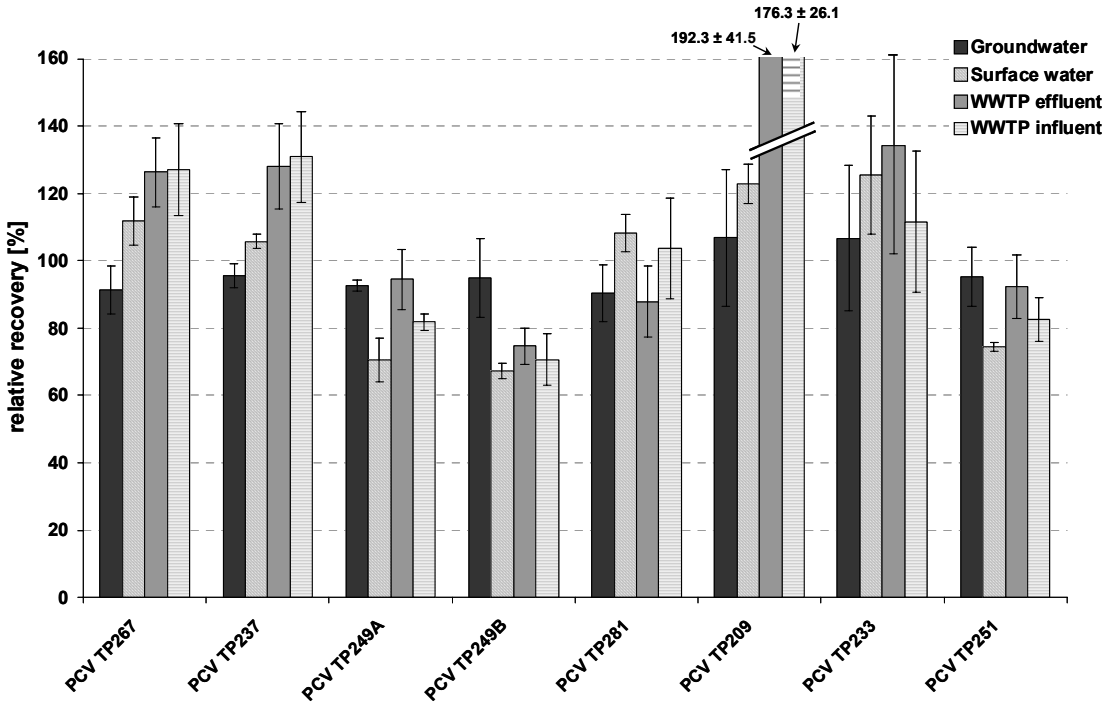


Figure S23. Relative recoveries (mean ± 95% confidence interval) of PCV TPs in the investigated aqueous matrices (n=4). For all TPs an amount of 500 ng was spiked. Only results of optimal SPE conditions (see Table S3) are shown.

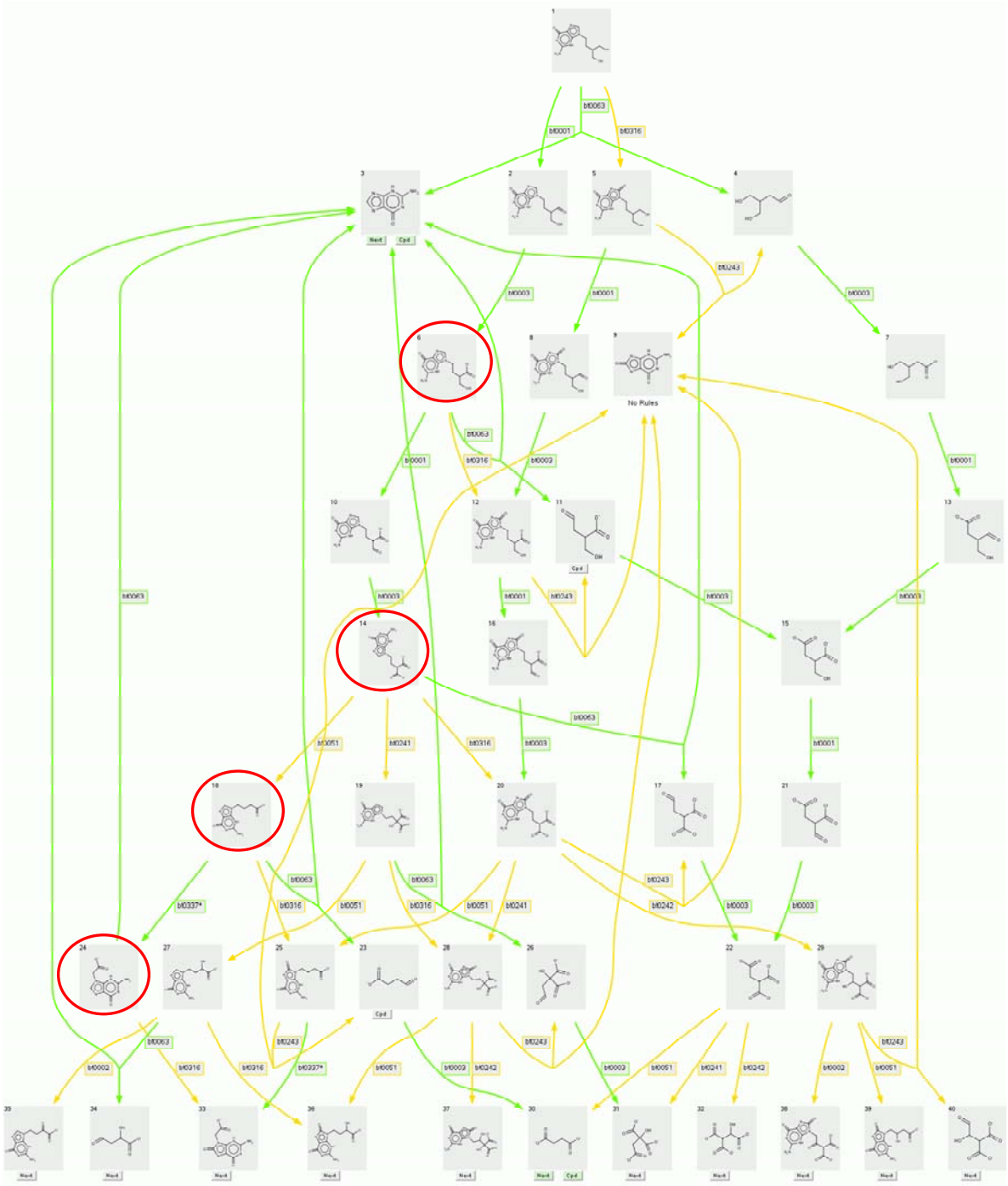


Figure S24. Predicted biodegradation pathway of penciclovir using the UM-PPS. Green arrows indicate reactions which are likely, yellow reactions those which are neutral. For more details see http://umbbd.msi.umn.edu/predict/. TPs observed in batch experiments are highlighted in red circles.

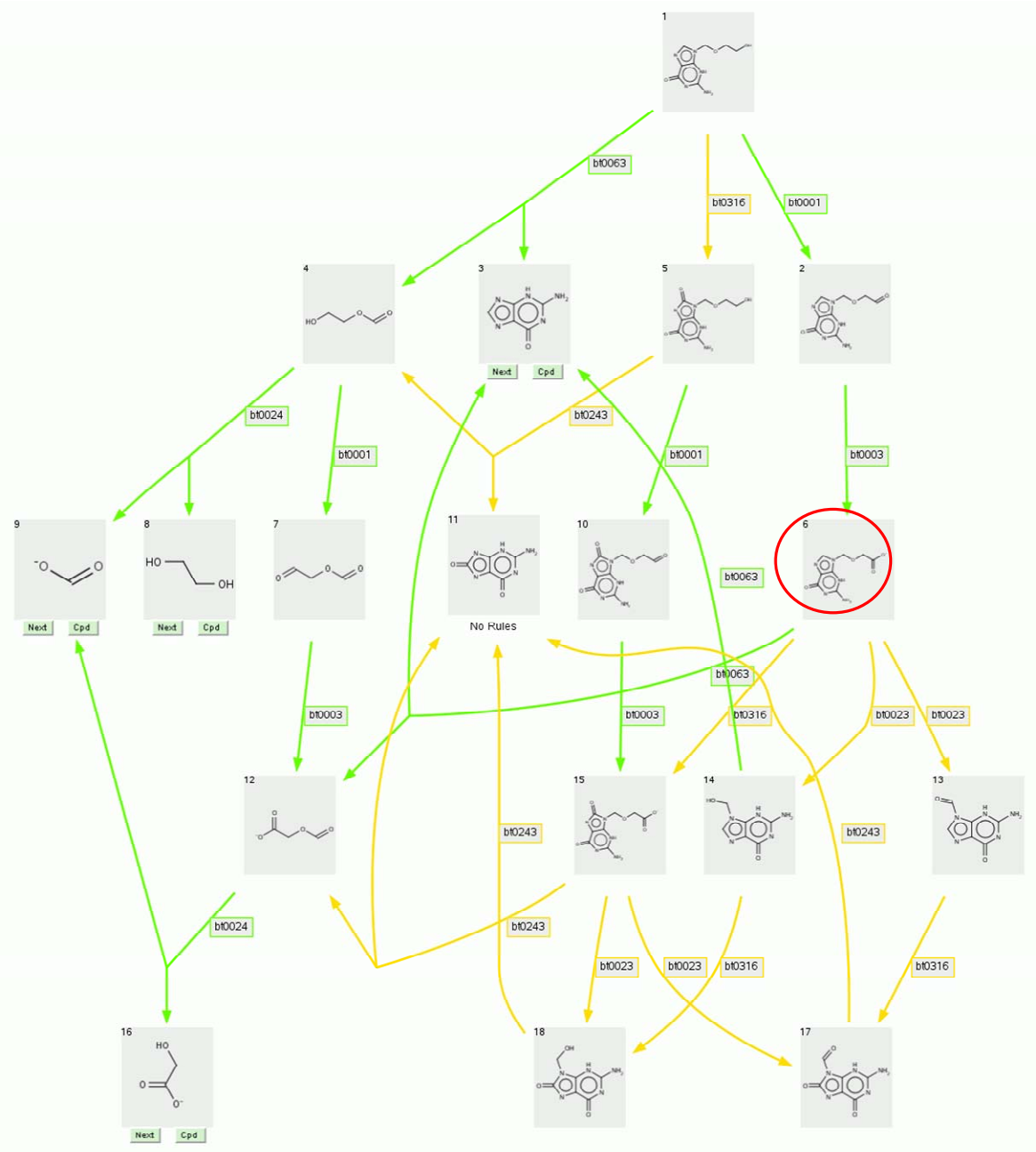


Figure S25. Predicted biodegradation pathway of acyclovir using the UM-PPS. Green arrows indicate reactions which are likely, yellow reactions those which are neutral. For more details see http://umbbd.msi.umn.edu/predict/. The TP observed in batch experiments is highlighted in the red circle.

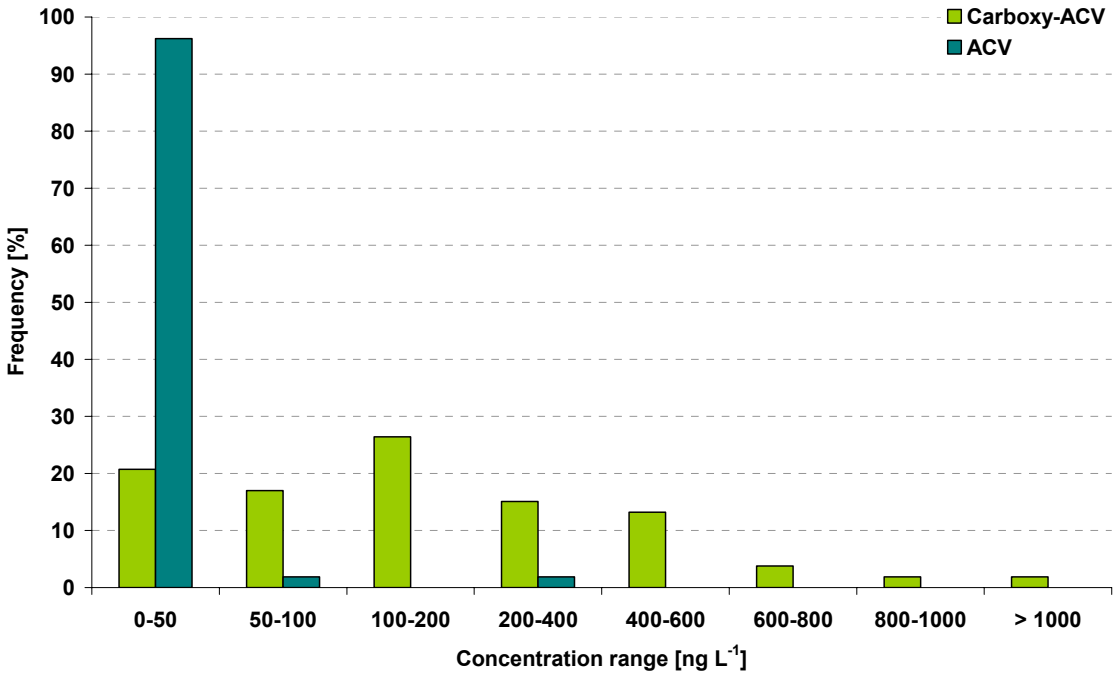


Figure S26. Frequency distribution plot of ACV and Carboxy-ACV concentrations in investigated surface water samples (n = 53).

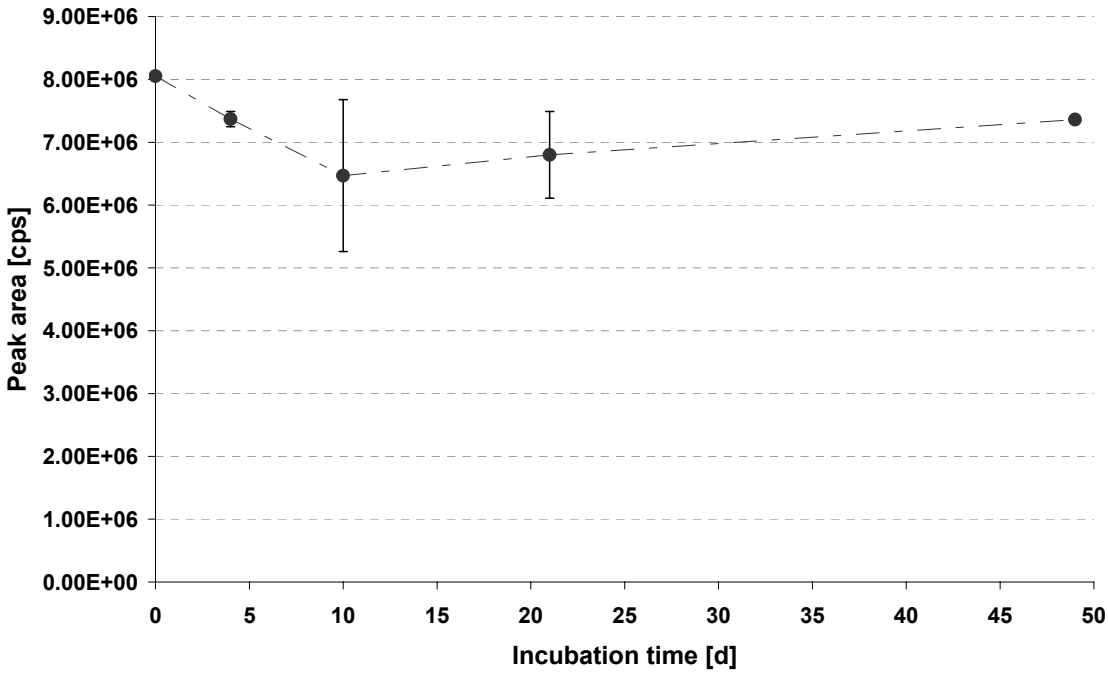


Figure S27. Peak area of Carboxy-ACV in soil batch systems over a total incubation time of 49 d using 80 g of soil and 400 mL groundwater. Details on the soil characteristics can be found in Yu et al. (*3*). Pressurized air and CO_2 were bubbled through the test vessels to maintain aerobic conditions at pH 7. The sludge concentration was 0.8 g_{SS} L^{-1}. Initial concentration of Carboxy-ACV in the batch systems was 500 ng mL^{-1}. Error bars indicate absolute deviations from mean (n=2).

References

(1) Engelhardt, I.; Piepenbrink, M.; Trauth, N.; Stadler, S.; Kludt, C.; Schulz, M.; Ternes, T. A., Quantification of Hydrodynamic Exchange Processes within the Hyporheic Zone via Environmental Tracers. *Journal of Hydrology* **(accepted)**.

(2) Prasse, C.; Schlusener, M. P.; Schulz, R.; Ternes, T. A., Antiviral Drugs in Wastewater and Surface Waters: A New Pharmaceutical Class of Environmental Relevance? *Environ. Sci. Technol.* **2010,** *44* (5), 1728-1735.

(3) Yu, L.; Fink, G.; Wintgens, T.; Melina, T.; Ternes, T. A., Sorption behavior of potential organic wastewater indicators with soils. *Water Res.* **2009,** *43* (4), 951-960.

A.3 SUPPORTING INFORMATION OF CHAPTER 5

Oxidation of the antiviral drug acyclovir and its biodegradation product carboxy-acyclovir with ozone – Kinetics and identification of oxidation products

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Content

Pages: 19 Tables: 3 Figures: 17

Tables

Figures

Chemicals and Standards

Acyclovir (ACV), guanosine and guanine were purchased from Sigma Aldrich (Schnelldorf, Germany) and acyclovir-d_4 was purchased from Campro Scientific (Berlin, Germany). Carboxy-acyclovir (carboxy-ACV) was isolated after incubation of ACV in activated sludge as described in Prasse et al.[1] The chemical structures of ACV, carboxy-ACV, guanine and guanosine are given in Figure S1. DMSO-d_6 (isotopic enrichment 99.96 %), used as NMR solvent, was purchased from Deutero GmbH (Kastellaun, Germany), tert-butanol (t-BuOH) was purchased from Merck (Darmstadt, Germany). Other solvents were picograde and purchased from LGC Promochem (Wesel, Germany). Chemicals were obtained from Carl Roth GmbH (Karlsruhe, Germany) unless noted otherwise.

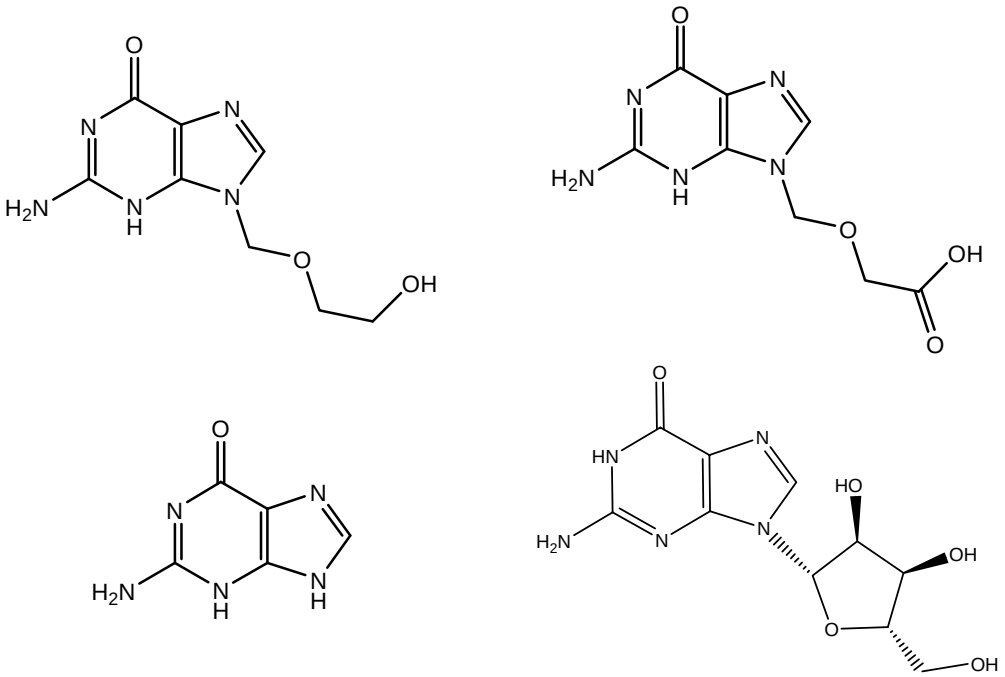


Fig. S1. Chemical structures of acyclovir (top, left), carboxy-acyclovir (top, right), guanine (bottom, left) and guanosine (bottom, right).

Analytical method for target analytes

ACV, carboxy-ACV, guanine and guanosine were analyzed by LC tandem MS in MRM mode using the two most intensive fragment ions (see Table S1). The HPLC system consisted of a G1367C autosampler, a G1312B binary HPLC pump, a G1379B degasser (all Agilent 1200 SL Series, Waldbronn, Germany) and a MistraSwitch column oven (MayLab Analytical Instruments, Vienna, Austria). For chromatographic separation a 4 µm Synergi Hydro-RP column (250 x 4 mm; Phenomenex, Aschaffenburg, Germany) was used. An aliquot of 20 µL of each sample were injected into the LC/MS/MS system using a mobile phase with 0.2 % HCOOH (v/v) (A) and methanol + 0.1% HCOOH (v/v). The gradient applied was as follows (percentage of A): 0 – 4 min, 100%; 7 min, 30%; 17 min, 10%; 18 min, 100%; 22 min 100%. The run time was 22 min, flow rate was 400 µL

min^{-1} and the column oven temperature was set to 40°C. The HPLC system was coupled to a API-4000 Qtrap (Applied Biosystems/MDS Sciex, Darmstadt, Germany). Analytes were quantified in multiple reaction monitoring (MRM) using electrospray ionization (ESI) with Turbo Ionspray in positive mode. Dwell time was 100 ms and ESI parameters were as follows: CUR, 35 psi; GS1, 45 psi; GS2, 45 psi; collision gas, high; ion spray voltage, 5,500 V; source temperature, 500°C; entrance potential, 10 V). The two most intense MRM transitions for each substance were monitored for identification and quantification of the analytes. Optimized MS parameters are given in Table S1.

Table S4. Precursor, product ions and optimized MS parameters used in LC/MS/MS detection

Substance	MRM 1	MRM 2	DP [V]	CE (MRM 1 / MRM 2) [eV]	CXP (MRM 1/ MRM 2) [eV]
Analytes					
Carboxy-Acyclovir (carboxy-ACV)	240.2 → 152.1	240.2 → 135.1	46	19 / 43	12 / 12
Acyclovir (ACV)	226.1 → 152.1	226.1 → 135.1	71	17 / 43	12 / 14
Guanine	152.1 → 135.1	152.1 → 110.0	46	27 / 29	10 / 8
Guanosine	284.1 → 152.1	284.1 → 135.1	36	21 / 55	12 /10
Internal Standards					
Acyclovir-d4	230.3 → 152.1	230.3 → 135.1	46	19 / 41	12 / 10

DP = Declustering potential; CE = Collision energy; CXP = Cell exit potential

Isolation of oxidation products (OPs)

Due to its environmental relevance and its close structural analogy to acyclovir, isolation of OPs was only performed for carboxy-ACV.[1] Therefore, carboxy-ACV was spiked to the test vessels attaining an initial concentration of 20 mg L^{-1}. An ozone: analyte ratio of 2:1 was used to ensure the complete oxidation of the parent molecule. Experiments were performed under ambient treatment conditions (pH 8) and at pH 3 to characterize the influence of the molecule speciation on OP formation. After the addition of ozone the mixture was allowed to react for about 2 h before residual ozone was removed by aeration of the reaction vessels with N_2. Afterwards, the samples were frozen (-25 °C) and lyophilized before individual OPs were fractionated via semi-preparative HPLC/UV.

To fractionate the OPs, an Agilent 1200 series HPLC system was used, equipped with an autosampler, column oven, quaternary pump, in-line degasser and a diode array detector. Isolation of individual OPs was achieved by chromatographic separation on a semi-preparative Hydro-RP column (250 x 10 mm; 4 µm; Phenomenex, Aschaffenburg, Germany) coupled to a semi-preparative Polar-RP column (250 x 10 mm; 4 µm, Phenomenex, Aschaffenburg, Germany). Aliquots of 500 µL were injected onto the columns. Individual fractions were collected with an automated sample collector (SF

2120 Fraction Collector, Techlab GmbH, Erkerode, Germany). As mobile phase, 0.2 % formic acid (A) and methanol + 0.1 % formic acid (B) were used. An eluent gradient was applied to achieve separation of the analytes. The percentage of (A) was changed linearly as follows: 0-15 min, 100%; 30 min, 30%; 33 min, 30%; 36 min, 90%. The total run time was 50 min. The flow rate was set to 2 mL min^{-1} and the column oven was operated at 40 °C.

An aliquot of each fraction was analyzed by HPLC/UV and LC-LTQ-Orbitrap MS to confirm the purity of the isolated OPs. Finally, each fraction was lyophilized to evaporate the solvents and the remaining water. The attained solid material of the OPs was used for NMR measurements and for the preparation of stock solutions for quantitative analyses.

HR-MS analysis of TPs via LTQ Orbitrap Velos ESI FT-MS

Chromatographic conditions were the same as for quantification by LC-tandem MS (see above). The ESI source parameters were set as follows: capillary temperature: 275 °C; capillary voltage 3.0 kV; heater temperature 300 °C; sheath gas flow rate 30 arbitrary units (AU); aux gas flow rate 10 AU; S-lens RF level 69%. Data dependent acquisition was used to conduct MS^2 and MS^3 spectra as follows: a full scan (100 – 500 m/z; positive mode) was performed followed by MS^2 and MS^3 scans for the 2 most intense ions with an intensity of >10000 counts per second (cps) and >1000 cps, respectively. CID (collision induced dissociation) with a normalized collision energy of 35% was used for fragmentation with an activation time of 10 ms. In addition, dynamic exclusion was applied (exclusion of masses for which 3 MS^n experiments have been performed; exclusion duration: 30 s) enabling also MS^n experiments for less abundant ions (e.g. during co-elution of different substances). For the elucidation of fragmentation patterns additional MS^n experiments were carried out by continuously infusing the compound solutions directly into the Orbitrap MS using a syringe pump.

NMR analysis

The structures of the transformation products were investigated on a Bruker Avance III 500 (11,7 T) with a 5 mm z-gradient BBFO ^1H/X probe. The proton and carbon spectra were measured in DMSO-d_6 at 298,3K and the spectra were referenced as follows: for the residual DMSO-(H)-d_5 $\delta(^1H)$ = 2,49 ppm and DMSO-d_6 $\delta(^{13}C)$ = 39,5 ppm.

A standard ^1H NMR spectrum needed between 32 and 128 transients attained with a 11 µs 90° pulse, 10000 Hz spectral width and a recycling delay between 1 and 10 s. ^{13}C NMR (125 MHz) experiments were obtained with ^1H decoupled (powergate decoupling using 30° degree flip angle) ^{13}C NMR acquisition methods and a spectral width of 25000 Hz.

The assignment was accomplished by $^1H,^1H$ COSY 2D method. The spectroscopic widths of the homo-nuclear 2D COSY experiments were typically 14000 Hz in both

dimension (f1 and f2) and the relaxation delay 1,2s. The 2D $^{1}H,^{13}C$-heteronuclear single quantum correlations (HSQC) were registered at a Bruker Avance III 500 system and Avance III 700. A 5 mm z-gradient TBI $^{1}H/^{31}P$/BB probe was used on the 700 MHz system. The spectroscopic width for the ^{1}H window was 10000 Hz (500MHz) or 14000 Hz (700 MHz) and for the ^{13}C 25000 Hz (125 MHz) or 35000 Hz (176 MHz).

All the 2D $^{1}H,^{13}C$-HSQC experiments were run by using 8192 points in f2 and 512 points in f1. Before Fourier transformation, the data were zero filled to 1024 points in f1 and multiplied by a window function (q-sine bell) in both dimension.

The following parameters were used to obtain optimal results: $^{1}J_{CH}$=145 Hz for optimizing observable intensities of cross peaks from one bond ^{1}H-^{13}C correlation, a relaxation delay of 2 s, while typical π/2 pulse width for ^{1}H were 11 µs (500 MHz) or 10,8 µs and ^{13}C and 9,5 µs (125 MHz) or 17 µs (176 MHz system).

Identification of OPs using HR-MS

OP formed at pH 8

Fig. S2 shows the MS fragmentation pattern of COFA. The exact masses, errors and double bound equivalents (RDB) of the parent ions and all fragments are given in Table S3.

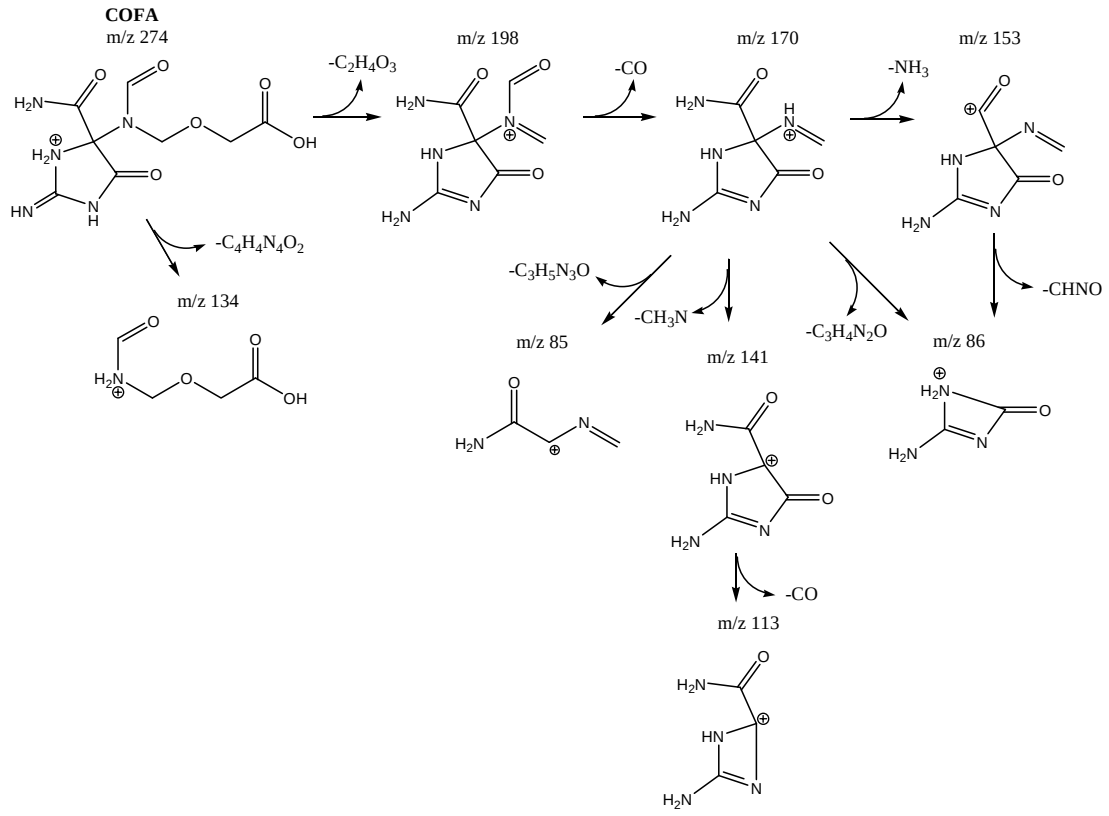


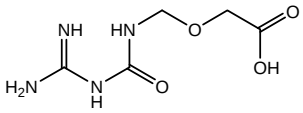
Fig. S2. MS fragmentation pattern of COFA.

OPs formed at pH 3

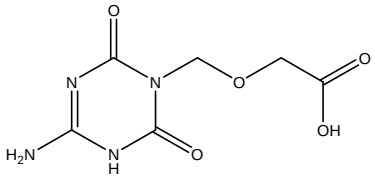
In contrast to pH 8, three different OPs were observed at pH 3, OP190, OP216 and OP245 (Table S2). However, as no NMR data was available structures are only tentative.

Table S2. Oxidation products from ozonation of CMMG at pH 3. Structures are only tentative as no NMR data was available.

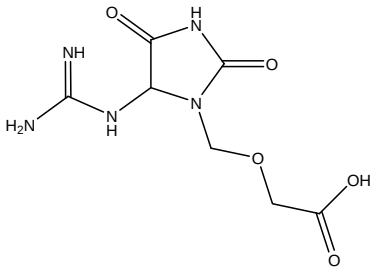
OP190



OP216



OP245



OP245. Eight different fragments were observed in MS^n spectra of OP245 (m/z 246) at m/z 188, m/z 170, m/z 141, m/z 128, m/z 113, m/z 99, m/z 98, m/z 72 (Fig. S2; Table S3). Even though m/z 170, m/z 141 and m/z 113 have also been observed for COFA, all other fragments are different. Further fragmentation of these fragments revealed that m/z 170 is derived from m/z 188 by the cleavage of water. Similar to COFA the results indicate that the side chain remained unchanged. However, in contrast to COFA a cleavage of the side chain at the ether is observed (fragment m/z 188) from which water can be eliminated leading to the formation of m/z 170. Based on these findings OP245 was identified as 5-carboxamido-5-formamido-guanidinohydantoin. Furthermore, also comparison of the MS spectrum with those of guanidinohydantoin formed from oxidation of guanine gave good agreement.[2, 3]

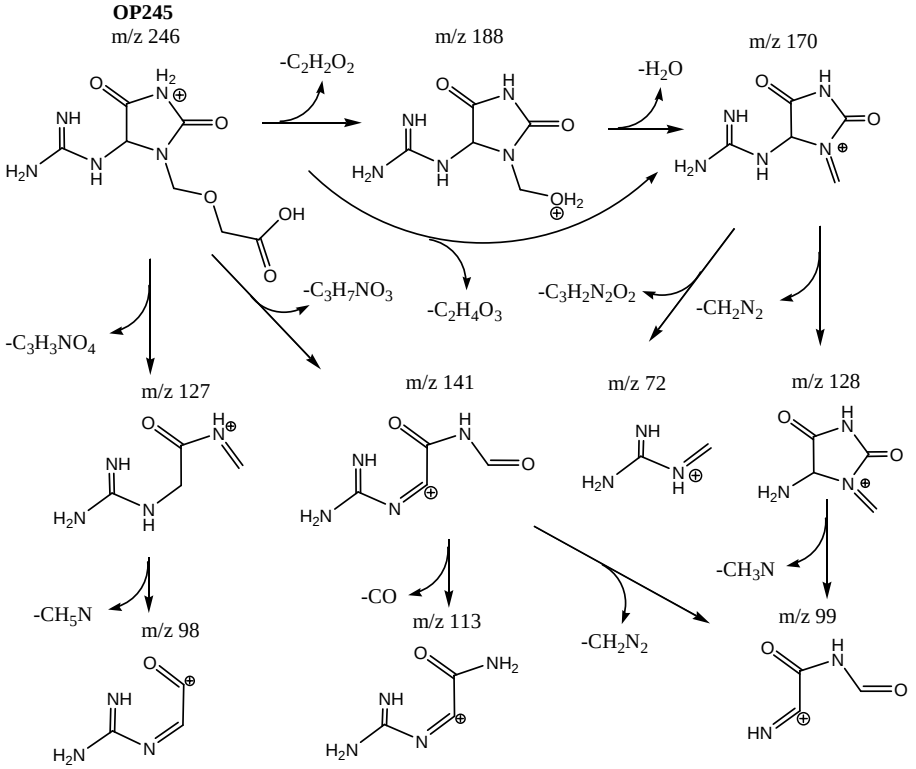


Fig. S3. MS fragmentation pattern of OP245. As no NMR data was available, the given chemical structure is only tentative.

OP216. 6 different fragments were observed in MS^2 - MS^4 spectra of OP216 (m/z 217) at m/z 141, m/z 129, m/z 112, m/z 98, m/z 86 and m/z 69 (Fig. S4; Table S3). m/z 112 and m/z 98 are derived from m/z 141. Furthermore, there is no cleavage of CO or H_2O indicating that neither an aldehyde nor a hydroxyl group is present in the molecule. Comparison of the MS spectrum of OP216 with those of the ammelide, a major degradation product of melamine, (corresponding to fragment m/z 129) gave good agreement with m/z 112, m/z 87 and m/z 86 being main MS fragments of this compound.[4, 5]

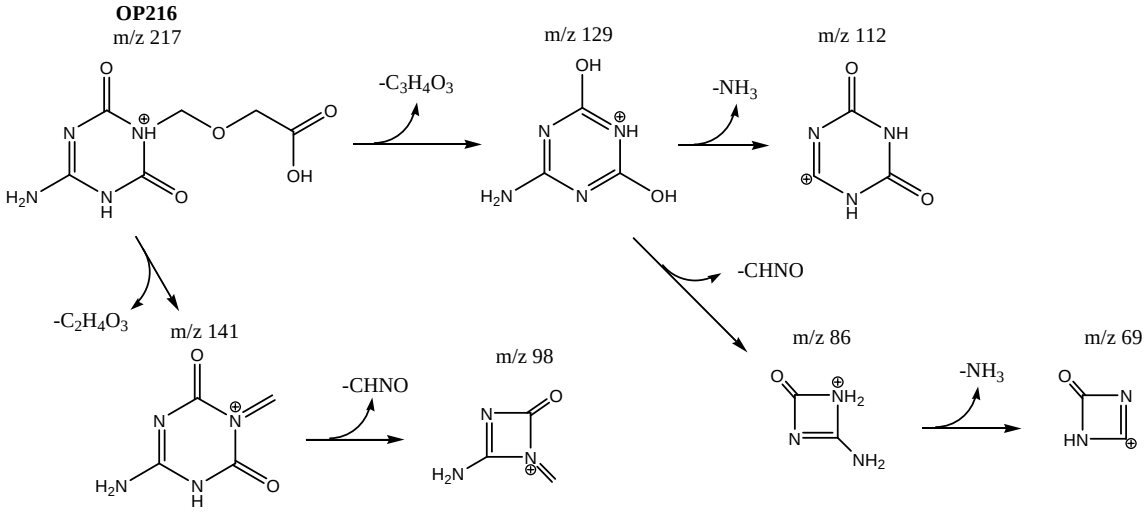


Fig. S4. MS fragmentation pattern of OP216. As no NMR data was available, the given chemical structure is only tentative.

OP190. Three different fragments were observed in the MS^2 - MS^3 spectra of OP190 (m/z 191) at m/z 115, m/z 86 and m/z 72 (Fig. S5; Table S3). Fragmentation of m/z 115 (MS^3) yielded m/z 86 by cleavage of CH_3N and m/z 72 by cleavage of CHNO. Similar to OP245 fragment m/z 72 indicates that the pyrimidine ring has been partially cleaved during ozonation.

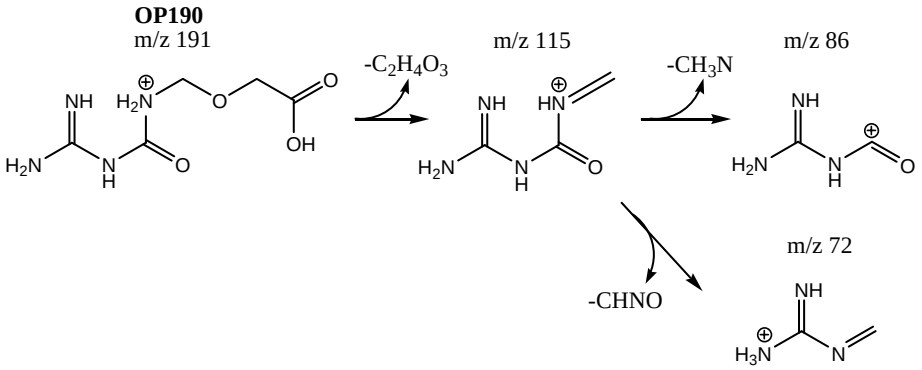


Fig. S5. MS fragmentation pattern of OP190. As no NMR data was available, the given chemical structure is only tentative.

Table S3. Accurate mass measurements of the oxidation products formed during ozonation of CMMG at pH 8 and pH 3 using LTQ Orbitrap Velos Electrospray Ionization in positive ion mode.

	Formula	Measd. Mass	mass error [ppm][a]	RDB	MS^n
		OP formed at pH 8			
COFA					
[M+H]+	$C_8H_{12}O_6N_5$	274.07739	-1.312	5.5	
	$C_6H_8O_3N_5$	198.06201	-0.786	5.5	MS^2
	$C_5H_8O_2N_5$	170.06703	-1.300	4.5	MS^3
	$C_5H_5O_2N_4$	153.04057	-0.862	5.5	MS^4
	$C_4H_5O_2N_5$	141.04050	-1.432	4.5	MS^4
	$C_4H_8O_4N$	134.04459	-1.449	1.5	MS^2
	$C_3H_5ON_4$	113.04554	-2.188	3.5	MS^5
	$C_2H_4ON_3$	86.03458	-3.584	2.5	MS^4/MS^5
	$C_3H_5ON_2$	85.03932	-3.755	2.5	MS^4
		OPs formed at pH 3			
OP190					
[M+H]+	$C_5H_{11}O_4N_4$	191.07738	-0.530	2.5	
	$C_3H_7ON_4$	115.06121	-1.976	2.5	MS^2
	$C_2H_4ON_3$	86.03457	-3.700	2.5	MS^3
	$C_2H_6N_3$	72.05528	-4.771	1.5	MS^3
OP216					
[M+H]+	$C_6H_9O_5N_4$	217.05658	-0.764	4.5	
	$C_4H_5O_2N_4$	141.04057	-0.936	4.5	MS^2
	$C_3H_5O_2N_4$	129.04052	-1.410	3.5	MS^2
	$C_2H_2O_2N_3$	112.01395	-1.811	4.5	MS^3
	$C_3H_4ON_3$	98.03465	-2.431	3.5	MS^3
	$C_2H_4ON_3$	86.03456	-3.816	2.5	MS^3
	C_2HON_2	69.00798	-5.206	3.5	MS^4
OP245					
[M+H]+	$C_7H_{12}O_5N_5$	246.08307	-0.914	4.5	
	$C_5H_{10}O_3N_5$	188.07762	-1.041	3.5	MS^2
	$C_5H_8O_2N_5$	170.06703	-1.300	4.5	MS^3
	$C_4H_5O_2N_4$	141.04048	-1.574	4.5	MS^2
	$C_4H_6O_2N_3$	128.04522	-1.820	3.5	MS^4
	$C_4H_7ON_4$	127.06125	-1.475	3.5	MS^2
	$C_3H_5ON_4$	113.04547	-2.807	3.5	MS^3
	$C_3H_3O_2N_2$	99.01859	-3.170	3.5	MS^3/MS^5
	$C_3H_4ON_3$	98.03458	-3.145	3.5	MS^3
	$C_2H_6N_3$	72.05526	-5.046	1.5	MS^4

[a] deviation from calculated mass

Formation of OP190, OP216 and OP245 with various amounts of ozone added

Fig. S6 shows the formation of the three OPs observed at pH3 with various ozone doses. As no standards were available only peak areas are given. For all three OPs a progressive increase was observed for experiments with and without t-BuOH as radical scavenger. However, it is indicated that the formation of OP216 and OP190 without the addition of t-BuOH takes place at lower analyte:O_3 ratios indicated from the steeper increase of the curves. In addition, OP216 and OP190 were not transformed further also at higher O_3 doses, whereas a strong decrease was observed for OP245 after CMMG was fully degraded by O_3. Even though the formation of $^{\bullet}$OH-radicals from O_3 at pH 3 is rather low, due to the stability of O_3 at low pH values, the stronger

decrease of OP245 in the experiment without the addition of t-BuOH indicates an involvement of $^{\bullet}OH$.

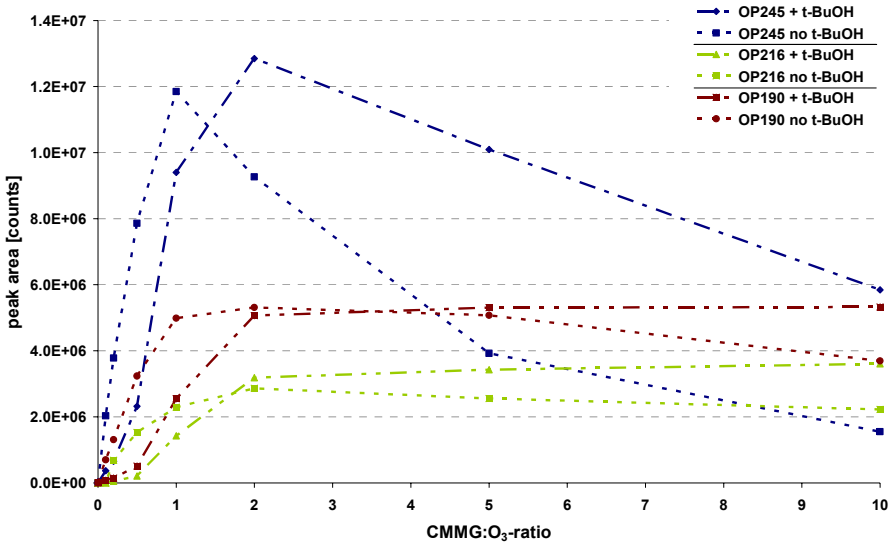


Fig. S6. Formation of OP190, OP216 and OP245 during ozonation of carboxy-ACV at pH 3 (phosphate buffer; 50 mM) with and without the addition of t-BuOH as radical scavenger. As no standards were available only peak areas are given.

Quantification of COFA in batch experiments

Due to the extensive formation of Na-adducts, $[M+H]^+$ and $[M+Na]^+$ were used for quantification. As no internal standard was available external calibrations were used by spiking different amounts of COFA into phosphate buffer (50 mM, pH 8; Fig S7) or WWTP effluent (DOC 10 mg L^{-1}, pH 7.8; Fig S8).

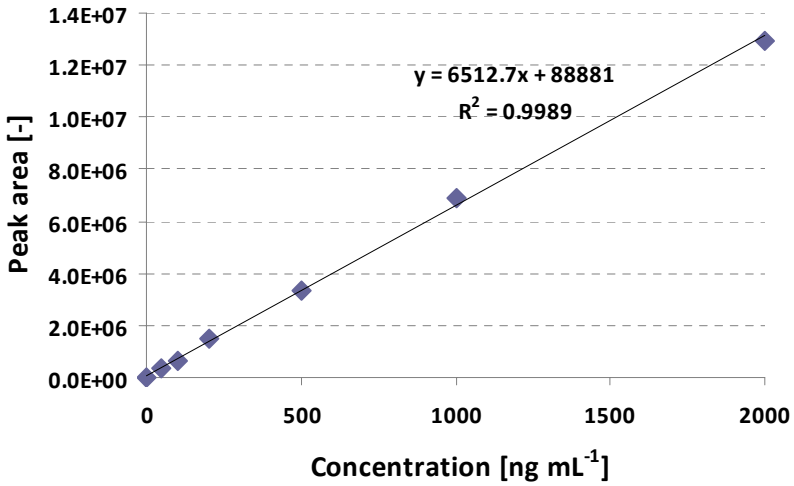


Fig. S7. External calibration curve of COFA in phosphate buffer (50 mM, pH 8). Due to the extensive formation of Na-adducts, the sum of [M+H]+ and [M+Na]+ were used for quantification.

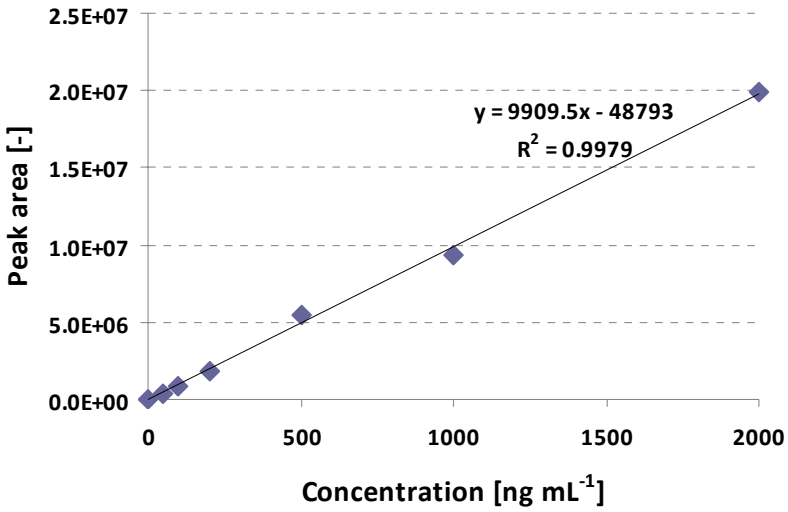


Fig. S8. External calibration curve of COFA in WWTP effluent (DOC 10 mg L^{-1}, pH 7.8). Due to the extensive formation of Na-adducts, the sum of $[M+H]^+$ and $[M+Na]^+$ were used for quantification.

Biodegradation of OP190, OP216 and OP245 during subsequent biological treatment

To investigate whether the OPs formed at pH3 can be further degraded in subsequent biological treatment, batch experiments with sewage sludge were performed. The sludge was taken from a conventional municipal WWTP. As the OPs were not available as reference substances they were spiked as a mixture with an approx. concentration of 200 µg L^{-1} (initial concentration of carboxy-ACV used for ozonation experiments). As can be seen from Fig. S9 a rapid decrease was observed for OP190. For OP216 a significant decrease was only observed after 48 h, whereas for OP245 no degradation was observed even after seven days.

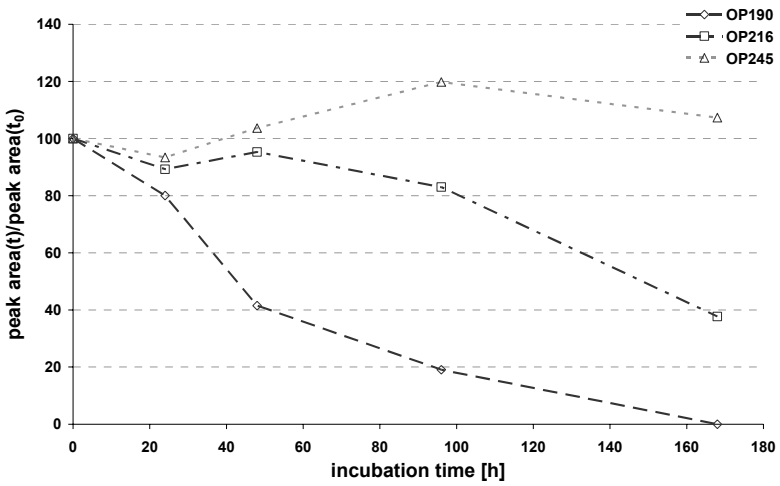


Fig. S9. Biodegradation of OP190, OP216 and OP245 in sewage sludge taken from the aerobic unit of a conventional municipal wastewater treatment plant. Total test duration was 168 h (7 days). Sludge was diluted to obtain an initial sludge concentration of 0.25 g_{TSS} L^{-1}. The exact initial concentration of OPs is unknown as no sufficient amounts could be isolated. Therefore, they were spiked as mixture with an estimated individual initial concentration of approx. 200 µg L^{-1}. The vessels were permanently aerated and stirred to maintain aerobic conditions. In addition, a small amount of CO_2 was added to the air to maintain a stable pH (pH 7).

Determination of degradation kinetics with ozone

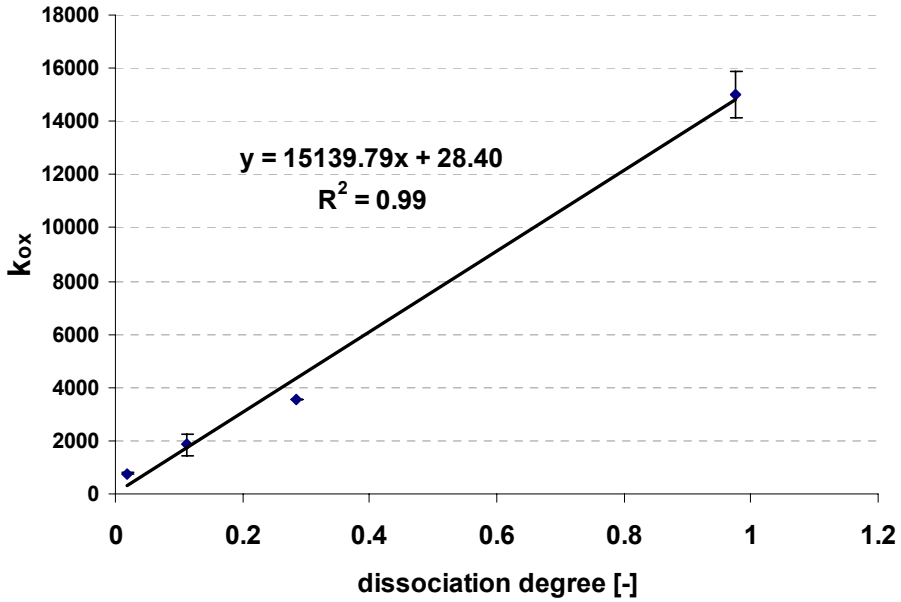


Fig. S10. Determination of $k_{ox,PH}$ using the results from experiments at pH 1.7, 2.5 and 3 (direct determination of degradation kinetics) and pH 5 (determined by competition kinetics).

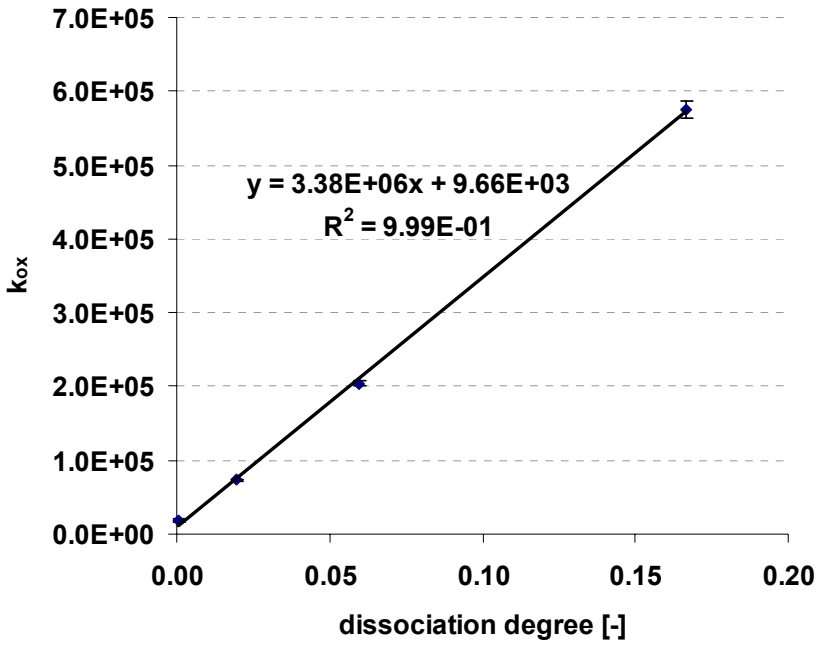


Fig. S11. Determination of $k_{ox,P-}$ using the results from experiments at pH 5, 6, 7.5, 8 and 8.5 (determined by competition kinetics).

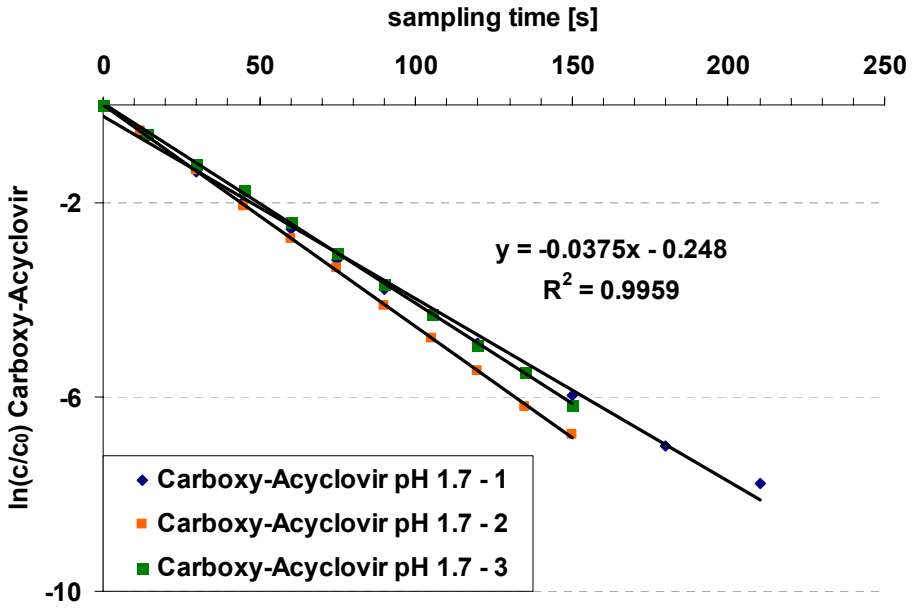


Fig. S12. Degradation kinetics of carboxy-acyclovir during ozonation at pH 1.7 determined in triplicate.

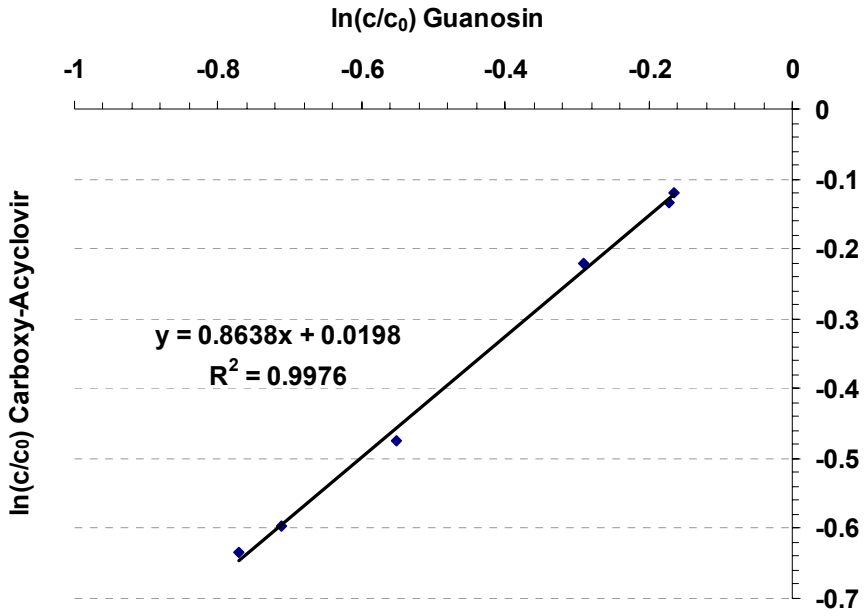


Fig. S13. Degradation kinetics of carboxy-acyclovir during ozonation at pH 8 determined using competition kinetics with guanosine as competitor.

NMR spectra of COFA

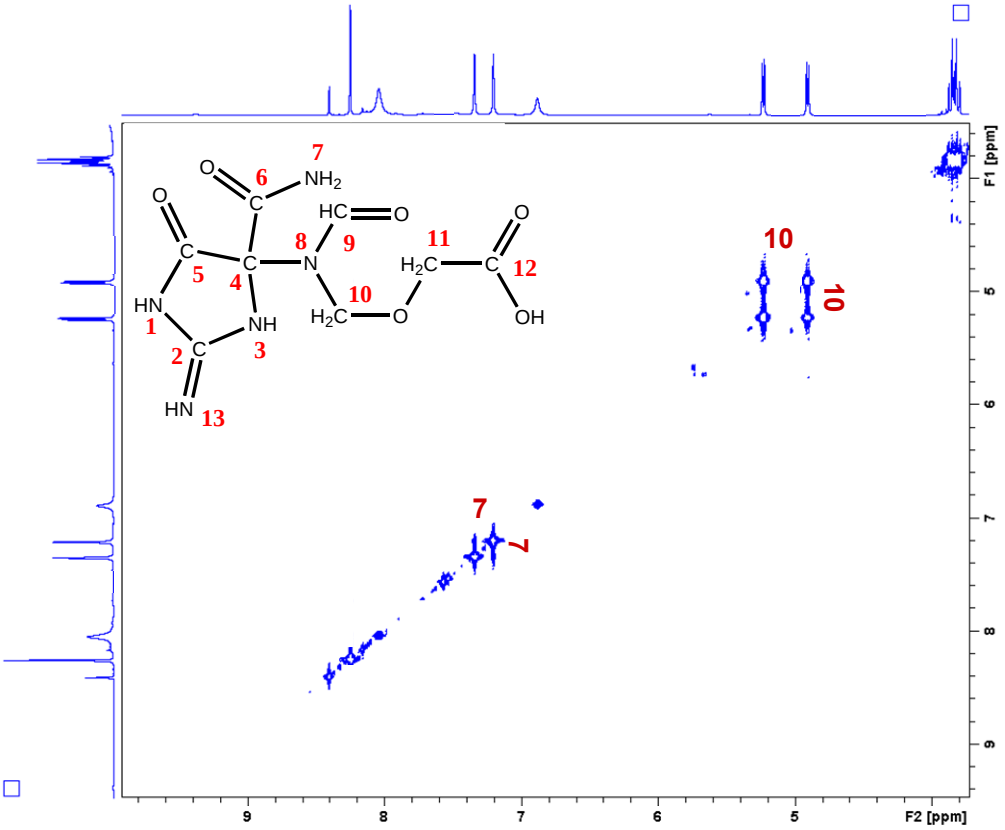


Fig. S14. ^{1}H,^{1}H-COSY spectrum of COFA.

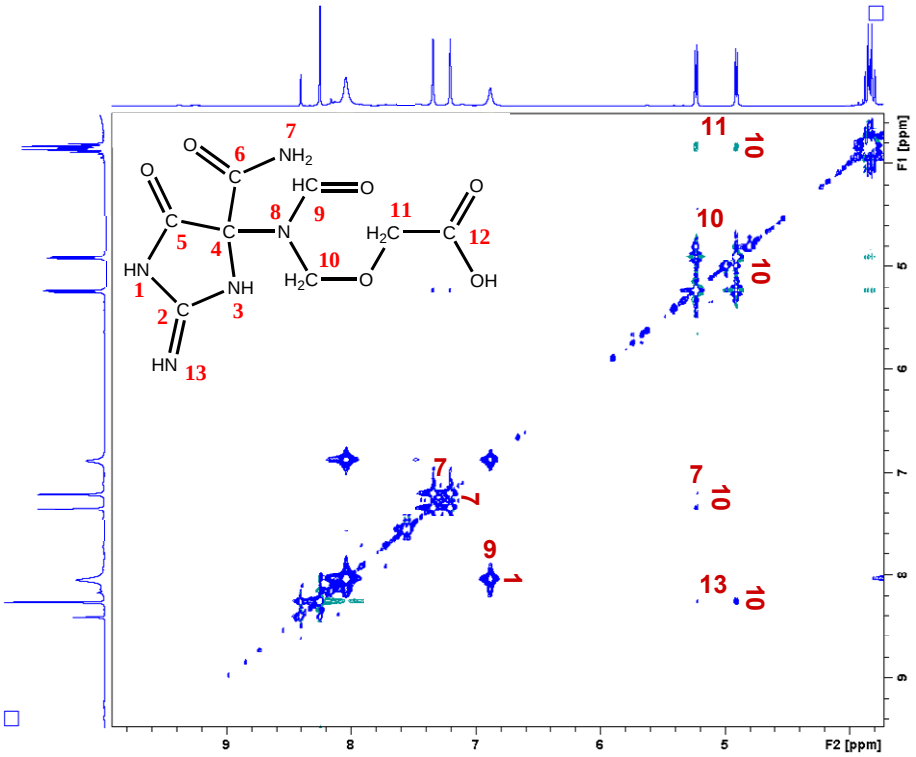


Fig. S15. $^{1}H,^{1}H$-NOESY spectrum of COFA.

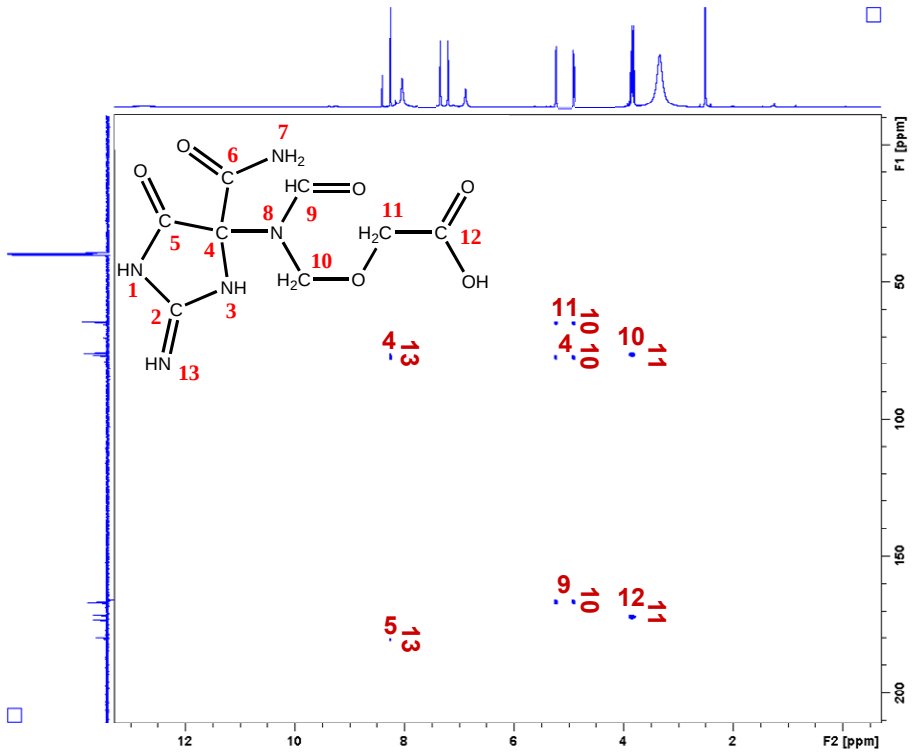


Fig. S16. $^{1}H,^{13}C$-HMBC spectrum of COFA.

Vibrio fischeri solution used for testing of bacterial toxicity

LB medium was used for growth of *V. fischeri* containing 30 g NaCl, 6.1 g $NaH_2PO_4*H_2O$, 2.75 g $K_2HPO_4*3H_2O$, 0.204 g $MgSO_4*7H_2O$, 0.5 g $(NH_4)_2HPO_4$, 3 mL glycerine, 5 g peptone from casein and 0.5 g yeast extract which were dissolved in one liter of ultrapure water. The pH of the medium was adjusted to pH 7 with NaOH. Afterwards, the medium was autoclaved for 20 min at 121°C and stored in the refrigerator until usage. The *V. fischeri* kits were purchased from Hach-Lange (Düsseldorf, Germany). The bacteria were stored frozen and rehydrated before testing by incubation in the medium with a rotary shaker (~ 120 U min^{-1}) for 48 h.

MS^2 spectra of COFA, acyclovir OP259, guanine OP186 and guanosine OP317

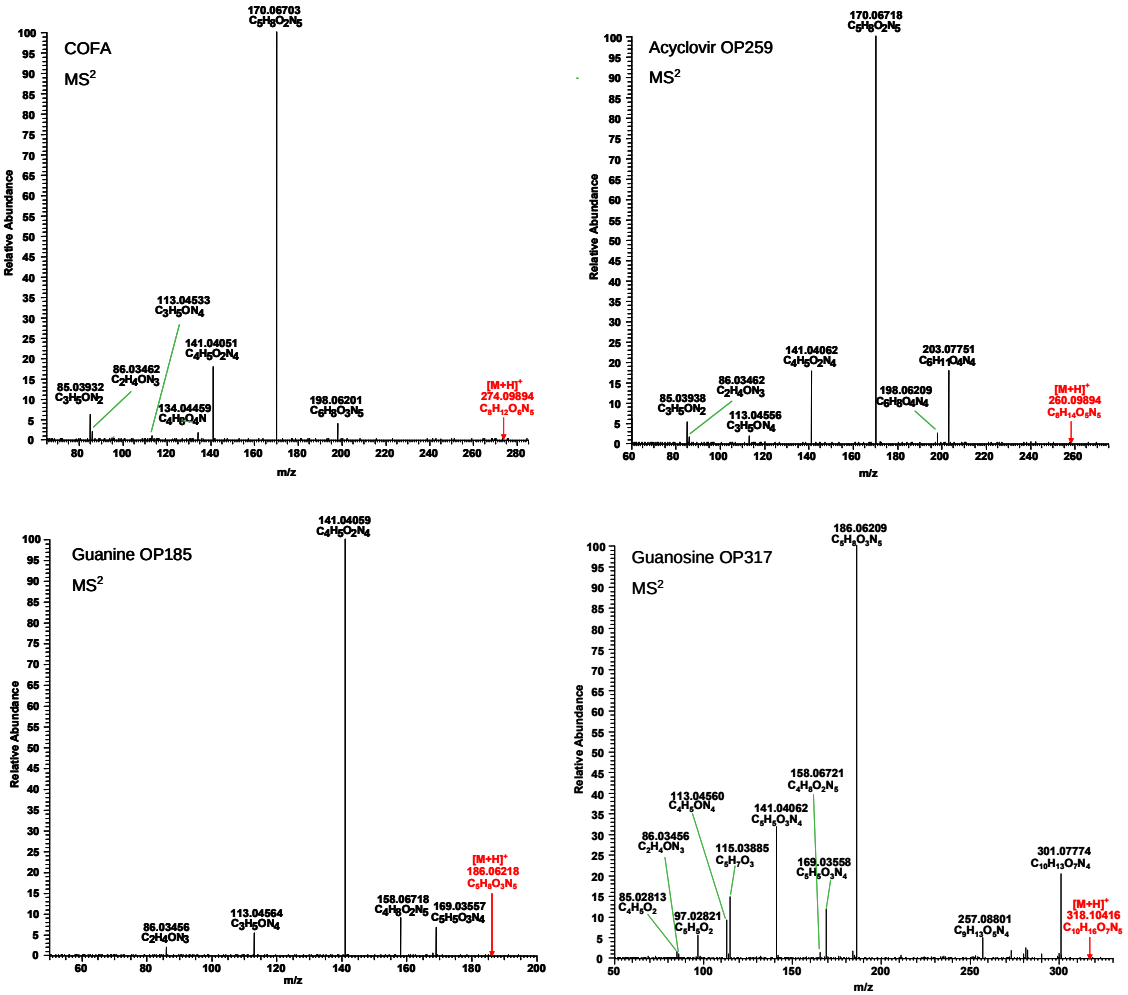


Fig. S17. MS^2 spectra of 2-imino-5-oxoimidazolidine oxidation products formed during ozonation of carboxy-ACV (COFA; top, left), acyclovir (acyclovir OP259; top, right), guanine (guanine OP185; bottom, left) and guanosine (guanosine OP317, bottom right) in WWTP effluent at ambient conditions (DOC 10 mg L^{-1}, pH 7.8). Higher energy collision dissociation (HCD) was used for fragmentation (normalized collision energy: 60.0; activation time: 0.1 ms).

References

(1) Prasse, C.; Wagner, M.; Schulz, R.; Ternes, T. A., Biotransformation of the Anti-viral Drugs Acyclovir and Penciclovir in Activated Sludge Treatment. Environ. Sci. Tech-nol. 2011, 45 (7), 2761-2769.

(2) Duarte, V.; Gasparutto, D.; Yamaguchi, L. F.; Ravanat, J. L.; Martinez, G. R.; Medeiros, M. H. G.; Di Mascio, P.; Cadet, J., Oxaluric acid as the major product of singlet oxygen-mediated oxidation of 8-oxo-7,8-dihydroguanine in DNA. Journal of the American Chemical Society 2000, 122 (51), 12622-12628.

(3) Ye, Y.; Muller, J. G.; Luo, W. C.; Mayne, C. L.; Shallop, A. J.; Jones, R. A.; Burrows, C. J., Formation of C-13-, N-15-, and O-18-labeled guanidinohydantoin from guanosine oxidation with singlet oxygen. Implications for structure and mechanism. Journal of the American Chemical Society 2003, 125 (46), 13926-13927.

(4) Vargha, M.; Takats, Z.; Marialigeti, K., Degradation of atrazine in a laboratory scale model system with Danube river sediment. Water Res. 2005, 39 (8), 1560-1568.

(5) Yu, H. A.; Tao, Y. F.; Chen, D. M.; Wang, Y. L.; Liu, Z. Y.; Pan, Y. H.; Huang, L. L.; Peng, D. P.; Dal, M. H.; Liu, Z. L., et al., Development of a high performance liquid chromatography method and a liquid chromatography-tandem mass spectrometry method with pressurized liquid extraction for simultaneous quantification and confir-mation of cyromazine, melamine and its metabolites in foods of animal origin. Anal. Chim. Acta 2010, 682 (1-2), 48-58.

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