Environmental Science & Technology Letters

Letter

pubs.acs.org/journal/estlcu

Toxification by Transformation in Conventional and Advanced Wastewater Treatment: The Antiviral Drug Acyclovir

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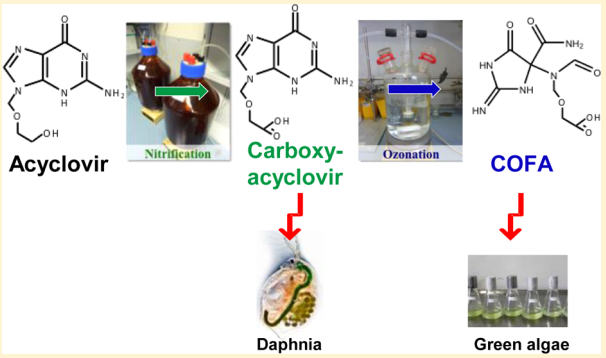
Supporting Information

ABSTRACT: Ozonation applied for advanced (waste)water treatment has a great potential to form polar transformation products (TPs) with often unknown toxicity. The antiviral drug acyclovir is transformed during biological wastewater treatment into carboxy-acyclovir. Ozone further transforms carboxy-acyclovir into *N*-(4-carbamoyl-2-imino-5-oxoimidazolidin)formamido-*n*-methoxy-acid (COFA). Both TPs have been detected in environmental samples and finished drinking water. Here, carboxy-acyclovir and COFA were produced at bench scale using treated wastewater and sewage sludge and were tested for aquatic toxicity in parallel with acyclovir. Carboxy-acyclovir was found to significantly reduce the level of reproduction of *Daphnia magna* (by 40% at 102 mg L^{-1}), and COFA inhibited the growth of green algae (E_rC_{10} of 14.1 mg L^{-1}); no toxicity was observed for acyclovir up to 100 mg L^{-1}. The predicted genotoxicity was not increased compared to that of the parent compound. In summary, the results highlight the importance of assessing the ecotoxicity of TPs formed during wastewater treatment, particularly in the case of ozonation.



INTRODUCTION

While knowledge of the environmental fate and effects of pharmaceuticals has improved considerably in recent years, similar information for their biotic and abiotic transformation products (TPs) formed naturally and in technical (waste)water treatment processes is widely lacking.[1−5] Whereas TPs are often reported to be less toxic than their parent compounds,[2,6−8] a study dealing with only pesticides and biocides indicated that in 20% of the cases the TPs exhibited an acute aquatic toxicity at least 3 times greater than that of the respective parent.[8] It remains unknown whether this finding can be transferred to the aquatic toxicity of TPs formed from other organic compounds such as pharmaceuticals. TPs that are formed in relevant amounts in the environment or by metabolic processes often have to be considered in the regulatory environmental risk assessment of the respective parent compound. In contrast, knowledge of the identity and potential hazard of TPs formed in conventional and advanced wastewater treatment processes is often not a standard requirement in regulatory environmental risk assessments. In particular, oxidation processes such as ozonation are known to be highly efficient with regard to primary degradation of a broad range of organic substances but may result in the formation of a great number of stable TPs with often unknown identity and toxicity[1,9−12] and genotoxic potential because of an increased reactivity.[13,14] The antiviral drug acyclovir (ACV), of which 45−75% is excreted by patients as unchanged compound,[15−17] is an example of a pharmaceutical with structurally identified TPs that are produced in wastewater treatment processes. Carboxy-acyclovir (C-ACV) is formed from ACV during nitrification and is transformed into *N*-(4-carbamoyl-2-imino-5-oxoimidazolidin)formamido-*n*-methoxy-acid (COFA) by ozonation. Because of its biological stability and high polarity, COFA cannot be removed by sand or activated carbon filtration. Both TPs have been detected in German river waters, wastewater treatment plant (WWTP) influents, effluents, and also finished drinking water.[18−20] The detection of ACV and its TPs in a broad range of environmental samples emphasizes the importance of the identification of TPs in the aquatic environment and in drinking water[2] and highlights the relevance of assessing (eco-)toxicological effects of these specific TPs.

Received: October 23, 2015
Revised: November 20, 2015
Accepted: November 23, 2015

DOI: 10.1021/acs.estlett.5b00291
Environ. Sci. Technol. Lett. XXXX, XXX, XXX−XXX

This study aimed to assess the aquatic ecotoxicity and to predict the genotoxic potential of ACV, C-ACV, and COFA. Sufficient quantities of TPs are prohibitively costly to synthesize, which often hinders their ecotoxicological testing. Here, the TPs were produced at laboratory scale using a setup in which treated wastewater and sewage sludge were incubated under aerobic conditions with ACV and ozonated thereafter. The whole laboratory treatment process was run in parallel without the addition of ACV to obtain controls that allowed separation of the effects of the TPs from effects of the treatments. Growth inhibition in green algae (*Raphidocelis subcapitata*), inhibition of the reproduction of the crustacean *Daphnia magna*, and survival of zebrafish embryos (*Danio rerio*) were used to assess aquatic toxicity at different trophic levels. Genotoxic potentials of ACV and its TPs were evaluated using the Distributed Structure-Searchable Toxicity (DSSTox) Database Network[21] and lazar (lazy structure−activity relationship) as the front end.[22]

METHODS

Biodegradation and Ozonation Experiments and Process Control Treatments. Biotransformation of ACV was achieved in two 10 L laboratory batch reactors. Sewage sludge from a nitrification unit of a German WWTP was diluted with treated effluent and continuously stirred and aerated with a mixture of air and CO_2 to maintain aerobic conditions and a stable pH of 7 ± 0.2. A freshly prepared stock solution of ACV dissolved in treated effluent was added, resulting in a final concentration of 200 mg L^{-1}. After complete transformation of ACV, the slurry was filtered and 2 L aliquots of the filtrate were subsequently ozonated. Biotransformation of ACV and oxidative transformation of C-ACV during ozonation were monitored using liquid chromatography−tandem mass spectrometry (LC−MS/MS).[19] The same setup was used for the process controls without adding ACV. Aliquot samples of all treatments were stored frozen (−20 °C) prior to testing in the different biotests. Further details can be found in the Supporting Information.

The terms C-ACV and COFA are used in the following to denote the treatments in which C-ACV and COFA were produced. The respective process controls are labeled as B (biological treatment) and B+O (biological treatment followed by ozonation).

Biotests. The following treatments were tested in parallel in each biotest: ACV, C-ACV, COFA, process control treatments B and B+O, and control treatment C0 consisting of culture medium of the respective test species. The parent compound ACV (CAS Registry Number 59277-89-3, Sigma-Aldrich, 99.6% pure) was dissolved directly in respective culture media and tested at 100 mg L^{-1}. Samples of C-ACV, COFA, B, and B+O were diluted with an equal amount of 2-fold concentrated culture medium of the respective biotest to ensure sufficient nutrient content for the test organisms. For detailed information about the biotests and used culture media, see the Supporting Information.

Algal Growth Inhibition Test. A static 72 h algal growth inhibition test was conducted with *Raphidocelis subcapitata* according to OECD 201. A second test was conducted in an identical way with a geometric dilution series (eight concentration levels with a spacing factor of 1.8) of the COFA treatment. The response variables biomass yield and growth rate after 72 h were evaluated for both tests.

***D. magna* Reproduction Test.** A semistatic 21 day reproduction test was conducted with *D. magna* according to OECD 211. The response variables survival, number of living offspring per surviving female within 21 days, and intrinsic rate of population growth were evaluated.

***D. rerio* Embryo Toxicity Test.** A static 96 h embryo toxicity test was conducted with embryos of in-house cultured zebrafish (*D. rerio*) according to OECD 236. The resulting response variable survival after 96 h was evaluated.

Analytical Measurements. Test solutions were sampled every week during the *Daphnia* test from corresponding fresh and aged media of every treatment and at the beginning and end of the first algal test. All samples were stored frozen at −20 °C until they were analyzed. ACV and C-ACV were analyzed using LC−MS/MS.[19] Concentrations of COFA were determined by the standard addition method using five spiking levels.

Statistical Analysis. Compliance with the assumptions of normal error distribution and homogeneous variances were confirmed visually and by Bartlett's, Cochran's, and Hartley's tests (at $\alpha = 0.01$), for the response variables algal yield, algal growth rate, *Daphnia* offspring, and *Daphnia* growth rate. Subsequently, a Tukey HSD test was performed in STATISTICA (version 12) to test for significant differences (two-sided, $\alpha = 0.05$) between treatments.

Using the software R and the *drc* package,[23] results for the response variables yield and growth rate determined in the second algal test were related to analytical measured concentrations of COFA and fitted by a three-parameter log−logistic model to estimate concentrations with 10 and 50% effects (EC_{10} and EC_{50}, respectively).

Genotoxicity Prediction. In the absence of valid data or sufficient amounts of substance for experimental testing, it is common practice to explore toxicological databases, expert systems, and other *in silico* approaches to assess the toxic potential of the chemicals of interest.[24] Here estimates of the genotoxic potential of ACV, C-ACV, and COFA were obtained with the help of the Distributed Structure-Searchable Toxicity (DSSTox) Database Network[21] via the lazar web interface. SMILES codes were generated from two-dimensional structures and inserted into the query form. The output provided qualitative estimates of the mutagenicity and carcinogenicity for mouse, rat, and hamster for the input structures. The decision was based on a fragment analysis and structure−activity-related comparisons.

RESULTS AND DISCUSSION

Biodegradation of ACV in the laboratory batch reactor was completed within 3 days, and the yield of C-ACV (approximately 200 mg L^{-1}) confirms complete transformation (molar mass balance of 106%). This is in good agreement with previous work showing that C-ACV is the only TP formed from ACV during biodegradation under aerobic conditions.[19] During ozonation, C-ACV was completely removed within 15 min and the final COFA concentration reached approximately 160 mg L^{-1}, demonstrating an incomplete transformation of 72% based on a molar mass balance, which was confirmed in measurements of biotest samples (Table 1), indicating the potential formation of other unidentified TP(s).

The analysis of C-ACV and COFA in the biotest samples confirmed the concentrations of TPs measured during the batch reaction. The concentrations measured in freshly prepared (initial) biotest solutions and those after exposure

DOI: 10.1021/acs.estlett.5b00291
Environ. Sci. Technol. Lett. XXXX, XXX, XXX−XXX

Table 1. Concentrations of ACV, C-ACV, and COFA in Samples of Biotest Treatments (ACV, dissolved in test medium; C-ACV, COFA, B, and B+O, wastewater samples of batch reactor treatments diluted 1:1 with test medium; control, test medium) at Test Start ($C_{initial}$) and after Exposure for 2−3 Days (C_{aged}) Given as Means (±standard deviation) of Measurements in the Algal and *Daphnia* Tests (n = 5 per sample)

sample	ACV (mg L^{-1})	C-ACV (mg L^{-1})	COFA (mg L^{-1})
$ACV_{initial}$	92.1 ± 6.5	<LOQ	<LOQ
ACV_{aged}	91.1 ± 9.0	<LOQ	<LOQ
$C\text{-}ACV_{initial}$	<LOQ	101.9 ± 14.1	0.2 ± 0.01
$C\text{-}ACV_{aged}$	<LOQ	105.3 ± 4.0	0.2 ± 0.02
$COFA_{initial}$	<LOQ	<LOQ	80.7 ± 3.0
$COFA_{aged}$	<LOQ	<LOQ	79.0 ± 5.6
B	<LOQ	0.001	<LOQ
B+O	<LOQ	<LOQ	<LOQ
control	<LOQ	<LOQ	<LOQ
LOQ	0.0001	0.0001	0.001

for 2−3 days (aged) confirmed that ACV and both TPs were stable during exposure (Table 1). ACV and COFA treatments contained neither of the two other analytes above their quantification limits, while the C-ACV treatment contained a small amount of COFA (<1%). Hence, effects observed in the biotests can be attributed directly to the presence of the individual TP (C-ACV or COFA) as long as statistically significant differences from the respective process control (B or B+O) are detected. Effects due to the presence of other chemicals or TPs can be identified by comparing the process and medium controls with each other.

The biotests fulfilled all validity criteria regarding water quality parameters (reported in the Supporting Information) and biological end points according to respective OECD guidelines. The only exception was the *D. rerio* embryo test, in which the hatching rate after 96 h was only 17% in the laboratory control instead of the required 80%. However, embryo survival after 96 h reached at least 95% in all wastewater and control treatments, and the required reduction of survival (20%) was achieved in the positive control. No sublethal effects were observed, indicating no acute fish toxicity of ACV and both TPs up to a concentration of ∼100 mg L^{-1}.

No mortality was observed in the *Daphnia* reproduction test. Reproduction and population growth rate of *D. magna* did not differ between the medium control and the ACV treatment (Figure 1), demonstrating that ACV exhibits no chronic *Daphnia* toxicity up to a concentration of 92.1 mg L^{-1}. Reproduction and population growth rate were significantly enhanced in the process control of the biological treatment (B) as well as in that of the biological treatment followed by ozonation (B+O) compared to the medium control. This effect may be attributed to better food conditions resulting from the bacterial load provided by the biological treatment. Reproduction and population growth rate were significantly reduced in the C-ACV treatment compared to the respective process control treatment B (by 39.9 and 22.4%, respectively) and the laboratory control. This indicates a significant increase in *Daphnia* toxicity of C-ACV compared to that of the parent. No significant differences occurred between COFA and the B+O treatment, indicating that COFA was not toxic to *Daphnia*.

Algal yield and growth rate were significantly inhibited in the COFA treatment compared to all other treatments, which did

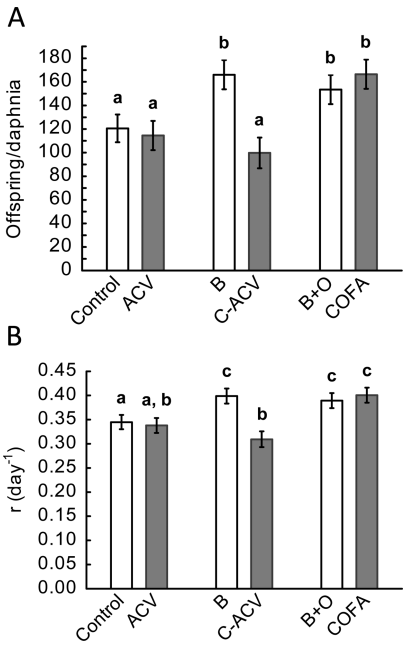


Figure 1. Reproduction measured as living offspring per female within 21 days (A) and intrinsic rate of population increase per day, r (B), of *D. magna* exposed to ACV, its transformation products present in the C-ACV and COFA treatments, the respective process control treatments B and B+O, and the medium control (M4). Shown are means with their 95% confidence intervals (n = 4 per treatment). Identical letters denote treatments that did not significantly differ from each other (Tukey HSD test; α = 0.05).

not differ among each other or from the (process) controls (Figure 2A,B). The toxicity of COFA toward algae was confirmed in the second test where an inhibition of yield and growth rate by 91.4 and 43.9%, respectively, was observed at the highest tested COFA concentration compared to the process control treatment B+O (Figure 2C,D). The EC_{10} (95% confidence interval) of COFA was estimated to be 4.12 (2.48−5.77) and 14.11 (11.17−17.06) mg L^{-1} for yield and growth rate, respectively. The EC_{50} was estimated to be 18.15 (15.44−20.87) and 101.57 (90.96−112.19) mg L^{-1} for yield and growth rate, respectively.

Effects of biologically active substances may be caused by specific receptor ligand interactions because even minor molecular modification of the active moiety of the molecule may lead to an altered toxicity in comparison with that of the parent compound.[8] The only structural alteration occurring due to the transformation of ACV to C-ACV is the formation of a carboxylic acid, while the transformation of C-ACV to COFA (see the graphical abstract) leads to a considerably different chemical structure. The possibility that these alterations are responsible for the observed toxicity of C-ACV to *Daphnia* and that of COFA to algae cannot be excluded. However, the observed toxicities in both cases are comparatively low, indicating an unspecific toxicity rather than a specific mode of action. The toxicity of COFA against *R. subcapitata* may furthermore be a toxicokinetic effect of ion trapping of the charged substance species inside the algal cells.[25]

Because of the incomplete mass balance for COFA, the possibility that other unidentified, minor TP(s) were formed from C-ACV during ozonation, which contributed to the observed algal toxicity, cannot be excluded. If we speculate that one other unidentified TP (produced at the remaining 28% of

DOI: 10.1021/acs.estlett.5b00291
Environ. Sci. Technol. Lett. XXXX, XXX, XXX−XXX

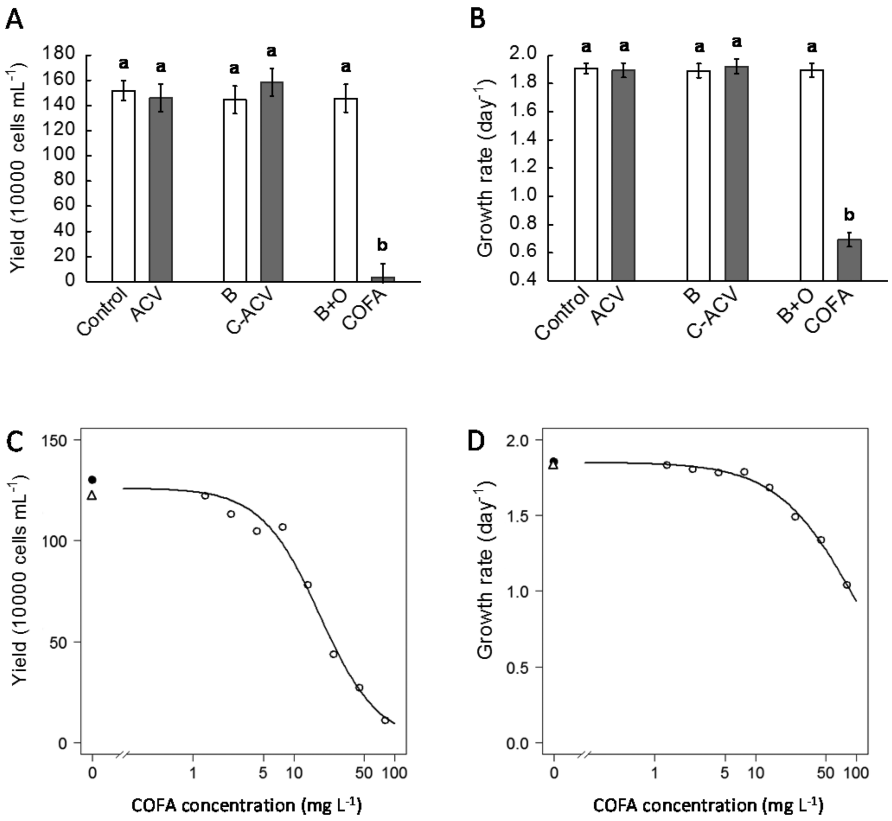


Figure 2. (A) Yield and (B) growth rate of *Raphidocelis subcapitata* after exposure for 72 h to ACV, its transformation products in the C-ACV and COFA treatments, the respective process control treatments B and B+O, and the control medium. Shown are mean responses with their 95% confidence intervals. Identical letters denote treatments that did not significantly differ from each other (Tukey HSD test; $\alpha = 0.05$). Concentration–response curves for (C) yield and (D) growth rate of *R. subcapitata* after exposure for 72 h to dilutions of the COFA treatment (based on measured COFA concentrations). Shown are means per treatment fitted by a three-parameter log–logistic model: (△) laboratory control, (●) process control treatment (B+O), and (○) COFA treatments.

the mass balance) fully accounted for the observed algal toxicity, this TP must have had a toxicity considerably higher than that calculated here for COFA. Because no other TPs could be identified during ozonation of C-ACV using very sensitive analytical techniques,[20] it appears most likely that either several TPs were formed at low concentrations or that the mass balance could not be closed because of limitations of the analytical methods (e.g., purity of the analytical standard).

The *in silico* predictions comprising reviewed mutagenicity and carcinogenicity data for common laboratory mammalians and *in vitro* test systems (see the Supporting Information, Table S5) did not suggest, on the basis of present structural evidence, a genotoxic potential of C-ACV and COFA greater than that of the parent compound ACV. Hence, no toxification regarding these end points by transformation was observed, which reduces the concern that ozonation of C-ACV-containing waters and subsequent movement of COFA to drinking water resources poses a risk to human health.

With the successful laboratory batch-scale production and ecotoxicological testing of C-ACV and COFA, the study presented here demonstrates a suitable approach to assessing the ecotoxicity of TPs that are not commercially available in sufficient amounts and quality. While the observed toxicity at 100 mg of C-ACV L^{-1} toward *D. magna* and the relevant toxicity estimate of COFA (14.11 mg L^{-1}, EC_{10} of algal growth rate inhibition) do not indicate an unacceptable environmental risk when compared with measured environmental concentrations of ~2.4 μg L^{-1}[19] and 0.001 μg L^{-1},[26] respectively, the results underline the general importance of studying the toxicity of TPs, even if they are formed from parent compounds showing no aquatic toxicity such as ACV. Similar to some TPs of pesticides and biocides,[8] TPs of pharmaceuticals can be more toxic than their parent compound. Species-specific toxicity of co-occurring compounds may translate to unexpected effects at the ecosystem level, which highlights the importance of applying a test battery covering different taxonomic and trophic levels to reliably characterize the ecotoxicological potential of TPs.

The TP(s) formed during ozonation (most likely COFA) exhibited the greatest increase in toxicity, which confirms the previous concern about the potential of oxidation processes such as ozonation to produce toxic TPs.[27] While the degree of toxicity increase observed for COFA (a factor of at least 7 more toxic to algae than its pharmacologically active parent compound; no increase in genotoxic potential) does not render ozonation *per se* as unsuitable for final wastewater purification, this study serves as an example providing clear evidence and should be an alert to the potential negative effects of ozonation for the receiving environment as many other wastewater-born chemicals may similarly form TPs of greater toxicity during the treatment process.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.5b00291.

DOI: 10.1021/acs.estlett.5b00291
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Detailed information about biodegradation and ozonation experiments, methods of conducted biotests, prediction of genotoxicity, and details of statistical analysis (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study is part of the TransRisk project (02WRS1275F) and was supported by the German Federal Ministry of Education and Research (BMBF) within the framework "Risk Management of Emerging Compounds and Pathogens in the Water Cycle (RiSKWa)".

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