Carbamazepine Ozonation Byproducts: Toxicity in Zebrafish (Danio rerio) Embryos and Chemical Stability

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ABSTRACT: Carbamazepine (CBZ) is an anticonvulsant medication with highly persistent properties in the aquatic environment, where it has the potential to affect nontarget biota. Because CBZ and many other pharmaceuticals are not readily removed in conventional sewage treatment plants (STP), additional STP effluent treatment technologies are being evaluated and implemented. Whole effluent ozonation is a prospective method to remove pharmaceuticals such as CBZ, yet knowledge on the toxicity of CBZ ozonation byproducts (OBPs) is lacking. This study presents, for the first time, in vivo individual and mixture toxicity of four putative OBPs, that is, carbamazepine 10,11-epoxide, 10,11-Dihydrocarbamazepine, 1-(2-benzaldehyde)-4-hydro-(1H,3H)-quinazoline-2-one (BQM), and 1-(2-benzaldehyde)-(1H,3H)-quinazoline-2,4-dione (BQD) in developing zebrafish (Danio rerio) embryos. BQM and BQD were isolated from the ozonated solution as they were not commercially available. The study confirmed that the OBP mixture caused embryotoxic responses comparable to that of ozonated CBZ. Individual compound embryotoxicity assessment further revealed that BQM and BQD were the drivers of embryotoxicity. OBP chemical stability in ozone treated CBZ water solution during 2 week dark storage at 22 °C was also assessed. The OBP concentrations remained over time, except for BQD which decreased by 94%. Meanwhile, ozonated CBZ persistently induced embryotoxicity over 2 week storage, potentially illustrating environmental concern.

1. INTRODUCTION

Pharmaceutical residues in sewage treatment plant (STP) effluents are increasingly studied as they are suspected to have persistent and toxic properties in the aquatic environment. Carbamazepine (CBZ) is an anticonvulsant pharmaceutical mainly prescribed to patients suffering from epilepsy and also used to mediate symptoms of schizophrenia and bipolar disorder. First synthesized in 1953 by Swiss chemists, CBZ has become widely used and it is included in the World Health Organization’s (WHO) Model List of Essential Medicines. The mode of action for CBZ is through sodium channel blocking by the therapeutically active carbamazepine 10,11-epoxide (CBZ-EP) metabolite, reducing synaptic activity in the central nervous system. About 13% of the ingested CBZ dose is excreted in unmetabolized form, mainly fecal excretion.

The overall removal efficiency of CBZ in conventional STPs is only about 2%. CBZ is consequently detected in surface water environments in the ng to μg L⁻¹ concentration range. CBZ is furthermore not prone to photodegradation. Mass balance-based models have suggested that approximately 55 metric tons of CBZ has accumulated in the Baltic Sea since its introduction to the market because of its persistent properties. Bioaccumulation of CBZ has been observed in, for example, bivalves and fish. Adverse effects resulting from CBZ exposure at concentrations proximal to environmental levels have been demonstrated in aquatic invertebrates. Fish are however considerably less sensitive to CBZ, with acute and chronic effects reported. Nevertheless, the constant release of CBZ and many other persistent and bioaccumulative pharmaceuticals warrants attention and could well be hazardous to fish because of mixture effects in polluted areas.

Novel technologies with the aim to improve STP removal efficiencies of pharmaceuticals and other micropollutants are being investigated. Studies on ozonation as a tertiary STP effluent treatment technology have shown that CBZ can be removed by more than 90% However, ozonation of CBZ...
can cause increased toxicity as observed in zebrafish (Danio rerio) embryos, and cell-based in vitro assays. Because the CBZ molecule has a high degree of reactivity with ozone, several intermediaries, that is, ozonation byproducts (OBPs), are created. Two key OBPs, 1-(2-benzaldehyde)-4-hydroxy-10,11-Dihydrocarbamazepine (DI-CBZ) and the therapeutically active CBZ metabolite CBZ-EP, have been quantified after ozonation of CBZ.7,17 There are considerable knowledge gaps regarding the identities and toxic potential of pharmaceutical OBPs. This study therefore aimed at evaluating individual and mixture toxicities of BQM, BQD, CBZ-EP, and DI-CBZ, which are formed following CBZ ozonation. Because BQM and BQD were unavailable as analytical standards at the time of the study, we proceeded to isolate them from ozonated CBZ. We hypothesized that one or more of these four OBPs could be the main drivers of ozonated CBZ embryotoxicity previously reported in zebrafish by us.17 Furthermore, the toxicity and composition of OBPs in STPs may change after release into the aquatic environment because of differences in the stability of the OBPs. We therefore sought to establish whether induced CBZ embryotoxicity postozonation, as well as OBP composition, would persist after storage of the OBPs in a water solution for up to 2 weeks.

2. MATERIALS AND METHODS

2.1. Chemicals. CBZ (CAS number 298-46-4, purity ≥ 98%), CBZ-EP (CAS number 36507-30-9, purity ≥ 98%), 10,11-Dihydro-10-hydroxycarbamazepine (DIOH-CBZ, CAS number 29331-92-8, purity ≥ 99%), and DI-CBZ (CAS number 3564-73-6, purity ≥ 99%) were purchased from Sigma-Aldrich (Sweden). Ethyl 3-amino-2-methoxy-1,3-benzozolate methanesulfonate salt (MS-222) was purchased from Sigma-Aldrich (Sweden). Ultrapure water was produced by a Milli-Q Advantage ultrapure water purification system (Millipore, Billerica, MA). Liquid chromatography–mass spectrometry (LC/MS) grade acetonitrile, methanol, and ammonium acetate were purchased from Sigma-Aldrich (St. Luis, MO, USA).

2.2. Ozonation of CBZ. A CBZ stock solution (25 mg L−1 nominal concentration) was prepared by dissolving CBZ in carbon-filtered tap water (22 °C, pH: 8.38 ± 0.02, conductivity: 453 ± 20 μS cm−1, alkalinity: 8 °dH, dissolved O2: 95 ± 4%) in a glass Erlenmeyer flask. Two 250 mL aliquots of CBZ were thereafter distributed to Erlenmeyer flasks. One aliquot was ozonated for 10 min (0.29 mg L−1 peak dissolved O3) using a lab-scale ozone generator (described in detail in the report by Pohl et al.1,15). Dissolved O3 concentrations were measured by the LCK310 Ozone cuvette test (0.05–2 mg L−1 measurement range) in a DR 3900 spectrophotometer (Hach, Loveland, Colorado, United States). A 60 mL grab sample was collected from the ozonated stock solution in a polypropylene centrifuge tube, snap-frozen in liquid nitrogen, and stored at ~80 °C for subsequent chemical analysis and transformation product isolation. The second CBZ aliquot was not ozonated. The Erlenmeyer flasks were thereafter stoppered and wrapped in aluminum foil for stability testing (Section 2.6).

2.3. Isolation of BQM and BQD. BQM and BQD were isolated from the ozonated CBZ solution for embryotoxicity testing. The grab sample (60 mL) was preconcentrated using solid-phase extraction (SPE) (Oasis HLB Plus short cartridge, 225 mg sorbent per cartridge, 60 μm particle size). The cartridges were activated with 5 mL methanol, rinsed with 5 mL Milli-Q water, and then dried for 1 min under vacuum. The samples (2 × 30 mL) were loaded onto two SPE cartridges and eluted with 2 × 5 mL methanol, and the volume was then reduced to 1 mL using a gentle stream of nitrogen gas.

Semipreparative LC was conducted on a Shimadzu LC-system consisting of two pumps (LC-10 ADvp), autoinjector (SIL-HTC), and a UV-detector (SPD-10 A). The separation was achieved on a reversed-phase C18 column (Vydac C18 218TP510) at a flow rate of 3 mL min−1. The mobile phase consisted of acetonitrile and Milli-Q water. The OBPs were eluted isocratically for 1 min with 15% acetonitrile followed by an increase in the acetonitrile content up to 80% over 15 min, after which it was lowered to 20% during 1 min. An equilibration time of 4 min was used between each injection. The UV-detector was set to operate at a wavelength (λ) of 285 nm. The two major peaks were identified according to the wavelengths specified by McDowell et al. (2005)28 and collected between the retention times of 8.60–9.20 (BQM) and 9.37–9.80 min (BQD). The injection of 100 μL was repeated 10 times. The peaks were collected in 25 mL jars. The solvent in each jar was let to evaporate in a fume hood at room temperature (22 °C) to dryness. The two isolated peaks were each transferred into an 18 mL glass test tube with a total of 10 mL methanol (rinsed 3 times in total). For BQM and BQD quantification in the final stock solutions, the extinction coefficient (ε) of CBZ was measured at 248 nm, and for the two OBP peaks the extinction coefficients were taken from McDowell et al. (2005)28 (Table 1). The Beer–Lambert law was used to calculate the concentrations of BQM and BQD.

\[ A = ε \times C \times l \] (1)

where A is the absorbance, ε the extinction coefficient, C the concentration, and l the length of the cuvette. Chemical concentration measurements indicated that a total stock solution content of 0.57 mg BQM, and 0.16 mg BQD were formed and isolated. The purity was >98% for BQM and >93% for BQD, based on the absorbance (Figure 1). The two isolated compounds (BQM and BQD) were used for subsequent embryotoxicity testing and as standard solutions

| compound     | RT (min) | M (g mol⁻¹) | ε  | λ (nm) | A     | C (mmol L⁻¹) | m (mg) |
|--------------|----------|-------------|----|--------|-------|-------------|-------|
| CBZ          | 10.7     | 236         | 11900/6 | 284   | 594998 | 0.1059      | 1.50  |
| BQM          | 8.8      | 250         | 35000/6 | 206   | 1876920| 0.2272      | 0.57  |
| BQD          | 9.4      | 266         | 28000/6 | 219   | 394158 | 0.0596      | 0.16  |

“Measured at 248 nm. “McDowell et al. (2005).28
for the determination of the actual concentrations in the toxicity test solutions.

2.4. Exposure Solutions. CBZ-EP and DI-CBZ exposure solutions were prepared from analytical grade powder mixed directly in tap water in Erlenmeyer flasks. BQM and BQD exposure solutions were prepared from isolated peaks solved in methanol. Aliquots of methanol-containing BQD and BQM, distributed in Erlenmeyer flasks, were allowed to fully evaporate inside a fume hood at 22 °C before tap water was added. All exposure solutions were thoroughly vortexed until compounds were completely dissolved. A mixture solution of BQM, BQD, CBZ-EP, and DI-CBZ was made by letting BQM and BQD methanol stock solutions evaporate before adding CBZ-EP and DI-CBZ exposure solution. All exposure solutions were prepared in carbon-filtered aerated tap water (pH: 8.38 ± 0.02, conductivity: 453 ± 20 mS cm⁻¹, alkalinity: 8.3°dH, dissolved O₂: 95 ± 4%). The same water was also used as a control treatment in each test. Physiochemical properties of each exposure solution did not deviate from that of tap water control.

2.5. Zebrafish Maintenance and OBP Embryotoxicity Tests. Zebrafish embryotoxicity test (ZFET) assays were performed under controlled ambient conditions (12:12 h light cycle, 26 ± 1 °C air temperature) according to previous methodology described in the report by Pohl et al. Adult laboratory-bred zebrafish were initiated to spawn directly before each exposure study (9 am to 11 am). Spawning was induced by placing fish (~5♂, ~5♀) in stainless steel spawning cages (5 mm mesh size) placed inside 10 L aquariums in the morning before lights were turned on at 9 am. Eggs collected from the spawning group displaying the highest fertilization success rate and the lowest proportion of abnormalities and coagulation were selected for ZFET.

The embryo exposure tests were static (the solutions were not changed during the test) and began ~3 h postfertilization (hpf) and continued until 144 hpf. Fertilized eggs were placed individually in 250 μL test solution (n = 16 per treatment) in round-bottomed 96-well microplates which then were covered by Parafilm M (Bemis Company, United States). During the exposure, heart rate (beats per min) was recorded at 48 hpf by manual counting in a stereomicroscope. Time until hatching (h) was assessed by automated photography (Canon EOS 500D) between 48 and 144 hpf (one photo per h). The proportion of malformations and lethality (expressed as % affected) was recorded at 144 hpf. After completion of the exposure test, the embryos were euthanized by exposure to a high dose of MS-222 (1 g L⁻¹).

2.6. Ozonated CBZ Stability and Embryotoxicity Tests. Aliquots (250 mL) of CBZ, ozonated CBZ, and control water were stored in the dark at room temperature (22 °C) for 2 weeks (336 h) in stoppered Erlenmeyer flasks wrapped in aluminum foil. Samples were collected from the three aliquots at 0, 1, 24, 168, and 336 h. One subset of samples (n = 3, v = 2 mL) were collected and stored in a freezer (−20 °C) for chemical analysis and another sample (n = 1, v = 40 mL) was used for zebrafish embryo exposure tests. The exposure tests (conducted as described in Section 2.5) commenced directly after sampling at the five storage timepoints. The proportion of affected (dead and malformed) embryos at 144 hpf was the only assessed endpoint because of time and logistical reasons, and as it was shown to be as sensitive as the other endpoints measured in our previous study.¹⁷

2.7. Chemical Analysis. The water samples were collected in triplicates from all exposure solutions at the start of each embryotoxicity test and kept frozen until chemical analysis (−20 °C). The samples were filtered using a regenerated cellulose syringe filter (0.22 μm pores) and spiked with the internal standard of CBZ (D10). The samples were analyzed using liquid chromatography tandem-mass spectrometry (LC–MS/MS) and a triple-stage quadrupole MS/MS TSQ Quantiva (Thermo Fisher Scientific, San Jose, CA, USA). An Acquity UPLC BEH-C18 column (Waters, 100 mm × 2.1 i.d., 1.7 μm particle size from Waters Corporation, Manchester, UK) was used as an analytical column. The injection volume was 10 μL for all samples. Heated electrospray ionization was used to ionize the target compounds. The spray voltage was set to static: positive ion (V) 3500.00. Nitrogen (purity > 99.999%) was used as a sheath gas (50 arbitrary units), auxiliary gas (15 arbitrary units), and sweep gas (2 arbitrary units). The vaporizer was heated to 400 °C and the capillary to 325 °C. Two selected reaction monitoring transitions were monitored for all analytes (Table S1). Data were evaluated using TraceFinder 3.3 software (Thermo Fisher Scientific, San Jose, CA, USA).

The internal standard method was used for the target compound quantification. The performance of the method was assessed concerning its linearity, limit of quantifications (LOQs), relative recovery, precision, blanks, and matrix effect. The linearity of the calibration curve was tested in the range from 0.001 to 10 mg L⁻¹. The calibration curve was measured twice, at the beginning and at the end of the sequence to check instrumental stability. The calibration was prepared in Milli-Q water. LOQs were calculated as half of the lowest calibration point in the calibration curve where the relative standard deviation of the average response factor was <30%. The peak area corresponding to this concentration was used to calculate LOQ for each individual compound in each sample. The

Figure 1. High performance liquid chromatography chromatogram of (a) peak 1 (BQM, 206 nm) and (b) peak 2 (BQD, 219 nm) after isolation.
The precision of the method was evaluated by the repeatability of the study. For this purpose, duplicates were conducted for every sample. The matrix effect was assessed for each compound, and corrections for ion suppression or enhancement were accomplished using matrix-matched standards. Matrix-matched standards were prepared from the ozonated water sample that was spiked with internal standard and native compounds at concentration levels of 0.01 and 0.1 mg L\(^{-1}\), respectively. The matrix effect was evaluated as the difference between the matrix-matched standards’ relative response factor and the average relative response factor obtained from the calibration curve. Quality control was confirmed by analysis of blank samples (Milli-Q and tap water) to assure that target analytes were not introduced from sampling or laboratory procedures and sample handling. No target analytes were detected in blank samples.

### 2.8. Statistical Analysis

R 3.6.0 software with a RStudio version 1.1.463 interface was used for statistical analysis.39,40 The R package ggplot was used for data plotting.31 Continuous data (i.e., heart rate and time until hatching) was checked and confirmed for normality and analyzed by one-way ANOVA with Dunnett’s post hoc test.42 Nonparametric data (i.e., proportion of affected embryos) was analyzed by Bonferroni-adjusted Fisher’s exact test. A p-value of p < 0.05 was considered as a significantly deviating effect as compared to the control in the statistical tests.

### 3. RESULTS AND DISCUSSION

#### 3.1. CBZ Ozone Removal Efficiency and OBP Formation

The CBZ molecule is prone to ozone degradation and will produce several intermediary OBPs (Figure 2). The present study was initiated by establishing the ozone removal efficiency of 17.5 mg L\(^{-1}\) CBZ ozonated 10 min in a laboratory-scale ozonation reactor (0.29 mg L\(^{-1}\) peak dissolved O\(_3\) concentration). The CBZ concentration was reduced by 82% following ozone ozonation, from 17.5 to 3.1 mg L\(^{-1}\). OBP formation (i.e., BQM, BQD, DI-CBZ, and CPZ-EP) from ozonated CBZ was also quantified (Table 2). BQD was the main product formed among the quantified OBPs, with a concentration of 5.5 mg L\(^{-1}\). The second most formed product was BQM (2.2 mg L\(^{-1}\)). BQM and BQD were quantified using isolated substances from ozonated CBZ as standards, meaning that the measured concentrations should only be indicative of actual concentrations. Previous studies have shown that BQM is the major initial OBP but its concentration decreases with increasing O\(_3\) concentration while BQD concentration, on the contrary, will increase with increasing O\(_3\).28,33,34 More specifically, McDowell et al.28 measured BQD and BQM concentrations in CBZ (0.85 mg L\(^{-1}\)) ozonated for up to 2.5 min (2.4 mg O\(_3\) min\(^{-1}\)), with the two OBP reaching approximately the same concentrations after 2.5 min. In the present study we measured higher concentrations of BQD than BQM after 10 min ozonation treatment (0.29 mg L\(^{-1}\) peak dissolved O\(_3\)), possibly because of the longer ozonation time allowing increased BQD formation.

CBZ-EP reached a concentration of 0.5 mg L\(^{-1}\) following ozonation. DI-CBZ concentrations were detected both in preozonated (0.04 mg L\(^{-1}\)) and postozonated (0.02 mg L\(^{-1}\)) CBZ samples. This could indicate DI-CBZ contamination in the CBZ used in the present study, as the DI-CBZ content in the preozonated CBZ solution was 0.02% (manufacturer reported CBZ purity ≥98%). An additional putative OBP, DIOH-CBZ, was included in the chemical analysis but not detected in any sample.

The ozone removal efficiency in the present study (82%) was lower than in our previous study, where an efficiency of >99% was recorded.17 Moreover, the DI-CBZ concentration following ozonation was lower in the present study (0.02 mg L\(^{-1}\)) than in our last study (0.2 mg L\(^{-1}\)).17 The difference in dissolved O\(_3\) concentration between the two studies could have led to diverging oxidation kinetics affecting CBZ removal and byproduct formation.5,36 Dissolved O\(_3\) measurements indicated that a higher O\(_3\) concentration was achieved in the present study (0.29 mg peak dissolved O\(_3\) L\(^{-1}\) after 10 min ozonation) using the same ozone reactor set-up as in the previous study (0.15 mg peak dissolved O\(_3\) L\(^{-1}\) after 10 min ozonation,17). The difference in O\(_3\) concentration could perhaps be explained by the use of a higher-capacity diffuser attached to the ozone generator. DI-CBZ formation has been shown to be inversely related to O\(_3\) concentration,17 which could explain why we quantified less DI-CBZ in the present study compared with our previous.

#### 3.2. Toxic Effects of Individual OBPs

The four CBZ OBPs (i.e., BQM, BQD, CBZ-EP, and DI-CBZ) were tested individually and in a mixture in ZFET assays. The toxicity endpoint heart rate (beats min\(^{-1}\)) at 48 hpf, time until hatching (hpf), and proportion of affected (dead and malformed) embryos at 144 hpf were measured (Figure 3). Three concentrations were tested in each study (Table 2). The single compound and mixture exposure concentrations were produced based on the OBP concentrations measured in Pohl et al.17 (for DI-CBZ and CBZ-EP) and the present study (for BQM and BQD). The intention was to design concentration ranges encompassing half (0.5×), equal (1×), and double (2×) that of the total OBP concentration quantified after ozonation of CBZ (Table 2). When comparing measured concentrations in the ozonated water (intended concentrations) with those of the 1× exposure solutions, DI-CBZ and BQM concentrations corresponded fairly well (Table 2). However, the measured concentrations for CBZ-EP and BQD in the 1× exposure solutions deviated from the intended concentrations, being about threefold higher for CBZ-EP and 2.5-fold lower for BQD. These deviations were likely caused by dilution or weighing errors. Besides, the fact that BQD was not stable during storage at room temperature (Figure 4c) may have added to the uncertainty of the analytical results for this OBP.

Exposure to BQM (Figure 3c) and BQD (Figure 3d) both resulted in decreased heart rate and prolonged hatching time. A clear concentration–response relationship was observed,
with the effect increasing with higher concentrations of both BQM and BQD. The proportion of dead and malformed embryos at 144 hpf was above control group levels in the 2× but not in the 1× concentrations of BQM or BQD. No effects in any endpoint were detected in the tested concentrations of either CBZ-EP (Figure 3e) or DI-CBZ (Figure 3f). CBZ-EP has been reported to be more toxic than CBZ to an aquatic invertebrate.37 In the present study, however, CBZ-EP and DI-CBZ concentrations at levels approximating that formed by CBZ ozonation did not elicit toxicity.

### 3.3. Mixture Toxicity of OBPs

One of the central focuses of the present study was to compare single compound and mixture toxicity. The rationale for assessing the mixture was to establish whether it would produce embryotoxic effects as observed in ozonated CBZ in our previous (Pohl et al.,17 Figure 3a) and present study (Figure 5a). Despite the uncertainty about BQM and BQD concentrations in exposure solutions after ozonation of CBZ in Pohl et al.17 (not screened for), and the deviation of measured CBZ-EP and BQD concentrations from the intended in the present study, the 1×...
mixture induced analogous effects (increased proportion of dead and malformed embryos at 144 hpf, decreased heart rate at 48 hpf, and prolonged hatching) as the ozonated CBZ solution in Pohl et al. and the present study (Figure 5a). The 1× mixture caused about 80% dead and malformed embryos at 144 hpf (Figure 3b), mainly manifested by yolk-sac edema (YSE) and pericardial edemas (PE) and lack of swim bladder inflation (SB). The 2× mixture induced lethality in all embryos already at 24 hpf, while the 0.5× mixture did not produce any statistically significant effects (Figure 3b). The hatching time delay observed in the previous study (Figure 3a, Pohl et al.) was however not reproduced in the present study for OBP mixture-exposed embryos (Figure 3b). This could well be because of a high degree of gross malformations and lethality (87.5%) occurring between 48 and 144 hpf, in the present study, leading to poor replication and thus low statistical power.

The measured concentrations in single compound and mixture exposure solutions in the present study did not markedly deviate, except for BQD which was measured at somewhat lower concentrations in the 1× and 2× single substance exposure solution than in the mixture solution (Figure 3). The overall comparisons of the toxicity of single compounds and mixtures were therefore not markedly impaired by large differences in concentrations in the different solutions (Figure 3). Taken together, the results of the chemical analyses show the importance of not depending on nominal concentrations when interpreting results in toxicity testing.

The present study is the first to test the toxic potencies of BQM and BQD in an in vivo assay. BQM and BQD induced toxic effects (i.e., reduced heart rate and prolonged hatching time) similar to what has been observed previously in zebrafish embryo-larvae exposed to ozonated CBZ (Figure 3a, Pohl et al.). The two other testedputative CBZ OBPs (i.e., CBZ-EP and DI-CBZ) did not affect these endpoints at relevant concentrations (Figure 3e,f). The results from the tests of the single OBPs strongly suggest that BQM and BQD were the drivers of the observed toxicity in the OBP mixture used in the present study. Toxicity data on CBZ OBPs is currently very limited. One study has attributed in vitro chromosomal damage following CBZ ozonation to BQD and BQM based on QSAR computer modeling.

3.4. Stability and Embryotoxicity of Pre- and Postozonated CBZ. Because CBZ has recalcitrant properties in the aquatic environment, we sought to also evaluate the stability of its main post OBPs. The aim was to study possible changes in CBZ OBP mixture composition and toxicity under controlled conditions. The concentrations of CBZ and four

Figure 4. Measured concentrations (mg L⁻¹) of target compounds in (a) tap water control, (b) carbamazepine, and (c) ozonated CBZ in tap water stored for 0, 1, 24, 168, and 336 h. The gray area signifies target compound LOQ ranges (maximum average LOQ: 0.0025 mg L⁻¹).

Figure 5. (a) Proportion of affected (dead and malformed) zebrafish embryo-larvae at 144 hpf after exposure to tap water control, 17.5 mg carbamazepine L⁻¹, and 17.5 mg carbamazepine L⁻¹ + O₃ stored for 0, 1, 168, and 336 h in the dark at 22 °C. Significant differences as compared to controls were indicated by Bonferroni-adjusted Fisher’s exact test (**p < 0.001). (b) Images representing embryo-larvae exposed to tap water control (normal), carbamazepine (normal), and carbamazepine + O₃ (PE, SB, YSE, and general growth retardation) at 144 hpf.
OBPs were measured in the three different treatment solutions (tap water control, CBZ, and ozonated CBZ) stored in the dark for 2 weeks at 22 °C (Figure 4). The same solutions were tested for embryotoxicity in parallel (Figure 5). Measurements of pH showed no difference between tap water (8.39 ± 0.01) and CBZ (8.39 ± 0.01) over the storage period. The pH of ozonated CBZ was initially lower than that of control and CBZ (8.18) but reached 8.37 after 336 h storage time. The CBZ concentration in nonozonated tap water remained stable (18.9 ± 1.0 mg L⁻¹) over the whole 2 week storage period (Figure 4b).

The concentration of BQD (5.5 mg L⁻¹), the main formed OBP screened for in the present study, decreased by 94% after 2 week storage reaching 0.29 mg L⁻¹ (Figure 4c). The second most formed OBP was BQM, with a concentration remaining relatively stable over the whole 2 week storage period (2.4 ± 0.3 mg L⁻¹). Both BQD and BQM in ozonated CBZ-spiked sewage effluent have been reported to continually degrade after 6 day storage in room temperature, transforming into their respective acid forms BaQM and BaQD, presumably by microbial processes. Only BQD was degraded following storage of ozonated CBZ-spiked tap water with presumably negligible microbial activity at room temperature for 2 weeks in the present study. CBZ-EP remained stable over the storage period (0.48 ± 0.06 mg L⁻¹). DI-CBZ concentrations also remained without significant reduction both in preozonated (0.04 ± 0.02 mg L⁻¹) and postozonated (0.02 ± 0.007 mg L⁻¹) CBZ samples.

The embryotoxicity (reported as % affected (dead and malformed embryos combined) after 6 d exposure) was tested at 0, 1, 168, and 336 h postozonation storage time of exposure mixtures (Figure 5a). The 24 h test was omitted because of excessive (>10%) spontaneous incidence of dead and malformed embryos in the tap water control group, compromising the results of that particular test. CBZ (17.5 mg L⁻¹) did not cause embryotoxicity at any tested storage time (Figure 4a). Exposure to ozonated CBZ, on the other hand, significantly induced embryotoxicity which remained throughout the whole storage period (Figure 5a). However, embryotoxicity decreased from ~95% at 0 h to ~75% at 336 h storage time, which is possibly linked to the BQD concentration reduction (94%) between 0 and 336 h storage time (Figure 4c). The embryotoxicity observed in the ozonated CBZ treatment group was mainly manifested by PE and YSEs appearing between 48 and 144 hpf (Figure 5b). Furthermore, embryos exposed to ozonated CBZ failed to hatch and properly inflate the swim bladder at 144 hpf at a larger extent than CBZ and tap water control. These results are in agreement with our previous study where CBZ ozonation under the same conditions resulted in equivalent malformations. Moreover, the same malformation types were predominant in embryos exposed to BQD, BQM, and the mixture (Figure 3). Residual ozone has been shown not to affect developing zebrafish embryos at the concentrations used in the present study.

In practice, the toxicity of CBZ following ozonation may be negated by applying, for example, filtration steps after ozonation. Subsequent biodegradation in a sand column has for instance been shown to be effective for the removal of BQD, BQM, and BaQM. The results of the present study, in general, reflect the situation occurring in a lab-scale reactor at CBZ concentrations 100–1000-fold above environmental relevance. Assessing the real-life situation in recipient surface waters, where sewage effluents have been treated with O₃, thus producing BQM and BQD, lies outside the scope of the present study. Other factors influencing chemical stability, including, for example, photodegradation, may also affect OBP concentrations over time. Nevertheless, because the elevated CBZ embryotoxicity after ozonation remains even after 2 week storage at room temperature, a continuous release of toxic OBPs (i.e., BQM) to the recipient may be problematic. Further research efforts should, therefore, focus on bioaccumulation properties and the long-term environmental effects of CBZ OBPs. Of particular interest is BQM, which displayed chemical stability and higher toxicity than the parent compound CBZ. The toxicity mechanisms of the OBPs should be unveiled as well. As we applied a target analysis approach, there is a possibility we might have overlooked other OBPs which may be responsible for additional toxicity. Consequently, the application of a nontarget screening approach will also be valuable in future studies.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b07100.

MS/MS parameters for a triple-stage quadrupole MS/TSQ Quantiva (PDF)

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**Notes**

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REFERENCES

(1) Richardson, S. D.; Ternes, T. A. Water Analysis: Emerging Contaminants and Current Issues. *Anal. Chem.* 2014, 86, 2813−2848.
(2) Shorvon, S.; Perucca, E.; Engel, J., Jr. *The Treatment of Epilepsy*, 4th ed.; Wiley, 2017; Vol. 2017.
(3) Schindler, W. New N-Heterocyclic Compounds. U.S. Patent 2,948,718, A, August 9, 1960.
(4) WHO. *WHO Model List of Essential Medicines*, 19th ed., 2015.
(5) Breton, H.; Coicgilio, M.; Bressolle, F.; Peyriere, H.; Blayac, J.; Hillarebuy, D. Liquid Chromatography−Electrospray Mass Spectrometry Determination of Carbamazepine, Oxcarbazepine and Eight of Their Metabolites in Human Plasma. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 2005, 828, 80−90.
(6) Kerr, B. M.; Thummel, K. E.; Wurden, C. J.; Klein, S. M.; Kroetz, D. L.; Gonzalez, F. J.; Levy, R. H. Human Liver Carbamazepine Metabolism: Role of CYP3A4 and CYP2C8 in 10,11-Epoxide Formation. *Biochem. Pharmacol.* 1994, 47, 1069−1077.
(7) McLean, M. J.; Macdonald, R. L. Carbamazepine and 10,11-Epoxycarbamazepine Produce Use- and Voltage-Dependent Limitation of Rapidly Firing Action Potentials of Mouse Central Neurons in Cell Culture. *J. Pharmacol. Exp. Ther.* 1986, 238, 727−738.
(8) Faigle, J. W.; Feldmann, K. F. Pharmacokinetic Data of Carbamazepine and Its Major Metabolites in Man. In *Clinical Pharmacology of Anti-Epileptic Drugs*; Schneider, H., Janz, D., Gardner-Thorpe, C., Meinardi, H., Sherwin, A. L., Eds.; Springer Berlin Heidelberg, 1975; pp 159−165.
(9) Björnling, S.; Ripzsám, M.; Haglund, P.; Lindberg, R. H.; Tykldm, M.; Eik, J. Pharmacological residues are widespread in Baltic Sea coastal and offshore waters—Screening for pharmaceuticals and modelling of environmental concentrations of carbamazepine. *Sci. Total Environ.* 2018, 633, 1496−1509.
(10) Heberer, T. Tracking Persistent Pharmaceutical Residues from Municipal Sewage to Drinking Water. *J. Hydrool.* 2002, 266, 175−189.
(11) Ternes, T. A. Occurrence of Drugs in German Sewage Treatment Plants and Rivers. *Water Res.* 1998, 32, 3245−3260.
(12) Tixier, C.; Singer, H. P.; Oellers, S.; Müller, S. R. Occurrence and Fate of Carbamazepine, Clofibric Acid, Diclofenac, Ibuprofen, Ketoprofen, and Naproxen in Surface Waters. *Environ. Sci. Technol.* 2003, 37, 1061−1068.
(13) Andreozzi, R.; Marotta, R.; Pinto, G.; Pollio, A. Carbamazepine in Water: Persistence in the Environment, Ozonation Treatment and Preliminary Assessment on Algal Toxicity. *Water Res.* 2002, 36, 2869−2877.
(14) Burket, S. R.; White, M.; Ramirez, A. J.; Stanley, J. K.; Banks, K. E.; Waller, W. T.; Chambless, C. K.; Brooks, W. B. Corbicula Fluminea Rapidly Accumulate Pharmaceuticals from an Effluent Dependent Urban Stream. *Chemosphere* 2019, 224, 873−883.
(15) Garcia, S. N.; Foster, M.; Constantine, L. A.; Huggett, D. B. Field and Laboratory Fish Tissue Accumulation of the Anti-Convulsant Drug Carbamazepine. *Ecotoxicol. Environ. Saf.* 2012, 84, 207−211.
(16) Ferrari, B.; Páxeus, N.; Giudice, R. L.; Pollio, A.; Garric, J. Ecotoxicological Impact of Pharmaceuticals Found in Treated Wastewaters: Study of Carbamazepine, Clofibric Acid, and Diclofenac. *Ecotoxicol. Environ. Saf.* 2003, 55, 359−370.
(17) Pohl, J.; Ahrens, L.; Carlsson, G.; Golovko, O.; Norrgren, L.; Weiss, J.; Órn, S. Embryotoxicity of Ozonated Diclofenac, Carbamazepine, and Oxazepam in Zebrafish (Danio Rerio). *Chemosphere* 2019, 225, 191−199.
(18) Kim, Y.; Choi, K.; Jung, J.; Park, S.; Kim, P.-G.; Park, J. Aquatic Toxicity of Acetaminophen, Carbamazepine, Cimetidine, Diltiazem and Six Major Sulfonamides, and Their Potential Ecological Risks in Korea. *Environ. Int.* 2007, 33, 370−375.
(19) Shao, Y.; Chen, Z.; Hollert, H.; Zhou, S.; Deutschmann, B.; Seiler, T.-B. Toxicity of 10 Organic Micropollutants and Their Mixture: Implications for Aquatic Risk Assessment. *Sci. Total Environ.* 2019, 666, 1273−1282.
(20) Prasse, C.; Stalter, D.; Schulte-Oehlmann, U.; Oehlmann, J.; Ternes, T. A. Spoil for Choice: A Critical Review on the Chemical and Biological Assessment of Current Wastewater Treatment Technologies. *Water Res.* 2015, 87, 237−270.
(21) Magdeburg, A.; Stalter, D.; Schlüssner, M.; Ternes, T.; Oehlmann, J. Evaluating the Efficiency of Advanced Wastewater Treatment: Target Analysis of Organic Contaminants and (Geno-)Toxicity Assessment Tell a Different Story. *Water Res.* 2014, 50, 35−47.
(22) Pohl, J.; Björnling, B.; Brodin, T.; Carlsson, G.; Fick, J.; Larsson, D. G. J.; Norrgren, L.; Örn, S. Effects of Ozonated Sewage Effluent on Reproduction and Behavioral Endpoints in Zebrafish (Danio Rerio). *Aquat. Toxicol.* 2018, 200, 93−101.
(23) Stalter, D.; Magdeburg, A.; Wei, M.; Knacker, T.; Oehlmann, J. Toxication or Detoxication? In Vivo Toxicity Assessment of Ozonation as Advanced Wastewater Treatment with the Rainbow Trout. *Water Res.* 2010, 44, 439−448.
(24) Dwivedi, K.; Rdrashetti, A. P.; Chakrabarti, T.; Pandey, R. A. Transformation Products of Carbamazepine (CBZ) After Ozonation and Their Toxicity Evaluation Using Pseudomonas Sp. Strain KSH-1 in Aqueous Matrices. *Indian J. Microbiol.* 2018, 58, 193−200.
(25) Han, Y.; Ma, M.; Li, N.; Hou, R.; Huang, C.; Oda, Y.; Wang, Z. Chlorination, Chloramination and Ozonation of Carbamazepine Enhance Cytotoxicity and Genotoxicity: Multi-Endpoint Evaluation and Identification of Its Genotoxic Transformation Products. *J. Hazard. Mater.* 2018, 342, 679−688.
(26) 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, 1016−1024.
(27) Hüblner, U.; Seiwert, B.; Reemtsma, T.; Jekel, M. Ozonation Products of Carbamazepine and Their Removal from Secondary Effluents by Soil Aquifer Treatment − Indications from Column Experiments. *Water Res.* 2014, 49, 34−43.
(28) 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, 8014−8022.
(29) R Core Team. *R: A Language and Environment for Statistical Computing*, R Foundation for Statistical Computing: Vienna, Austria, 2019.
(30) R Studio Team. *RStudio: Integrated Development for R*, R Studio Inc.: Boston, MA, 2019.
(31) Wichmann, H.; *Ggplot2: Elegant Graphics for Data Analysis*; Springer-Verlag: New York, 2016.
(32) Hothorn, T.; Bötz, F.; Westfall, P. Simultaneous Inference in General Parametric Models. *Biom. J.* 2008, 50, 346−363.
(33) Alharbi, S. K.; Price, W. E.; Kang, J.; Fujioka, T.; Nghiem, L. D.; Sener, M.; Ternes, T. A.; Noh, S. Effects of Ozonated Wastewater on Zooplankton Community Structure: Aggregated Results from Four Continuous Chronic Experiments. *Water Res.* 2013, 1273−1283.
(34) Breton, H.; Ahrens, L.; Carlsson, G.; Golovko, O.; Norrgren, L.; Weiss, J.; Örn, S. Embryotoxicity of Ozonated Diclofenac, Carbamazepine, and Oxazepam in Zebrafish (Danio Rerio). *Chemosphere* 2019, 225, 191−199.
(35) Lee, Y.; von Gunten, U. Oxidative Transformation of Micropollutants during Municipal Wastewater Treatment: Comparison of Kinetic Aspects of Selective (Chlorine, Chlorine Dioxide, FerrateVI, and Ozone) and Non-Selective Oxidants (Hydroxyl Radical). *Water Res.* 2010, 44, 555−566.
Heye, K.; Becker, D.; Lütke Eversloh, C.; Durmaz, V.; Ternes, T. A.; Oetken, M.; Oehlmann, J. Effects of Carbamazepine and Two of Its Metabolites on the Non-Biting Midge Chironomus Riparius in a Sediment Full Life Cycle Toxicity Test. *Water Res.* **2016**, *98*, 19–27.