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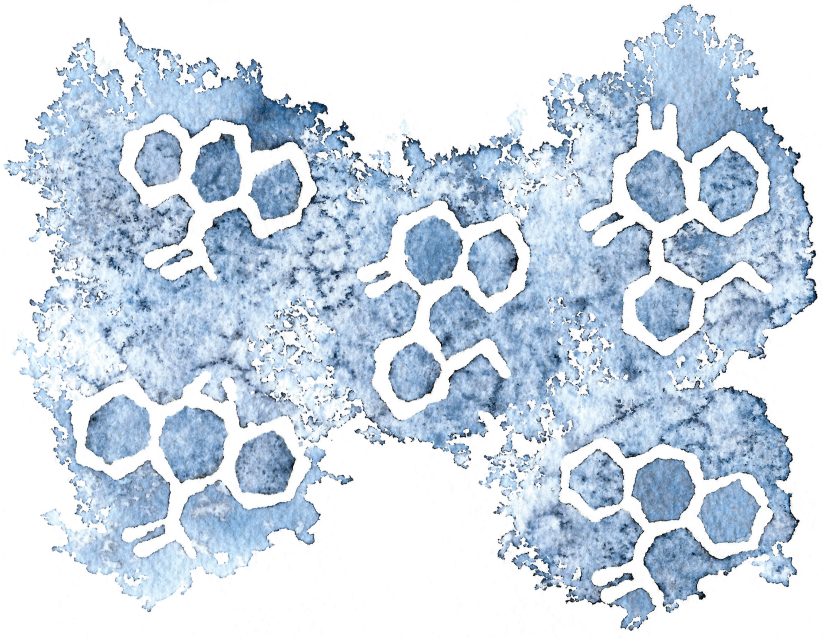

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Abstract

The aquatic environment is the main destination for many organic pollutants originating from human activity. Pharmaceuticals are significant contributors to this pollutant cocktail. One major route of pharmaceuticals into the aquatic environment is through discharges from sewage treatment plants (STPs) with inadequate pharmaceutical removal capacity. Adverse effects in aquatic organisms exposed to pharmaceuticals have been illustrated in a multitude of studies. Advanced sewage treatment technologies are therefore being evaluated as potential methods for improving pharmaceutical removal, for example whole-effluent ozonation. However, it remains important to assess how the ozonation treatment modulates the biological effect in aquatic organisms exposed to the STP effluent due to the formation of ozonation by-products (OBPs).

This thesis aimed to investigate outcomes of ozonation treatment of STP effluents with a special focus on pharmaceuticals, by combining chemical analysis and *in vivo* testing using zebrafish (*Danio rerio*). Firstly the effects of an ozonated STP effluent on reproduction and behavior of adult zebrafish were evaluated at a Swedish STP (Knivsta municipal STP) with a full-scale ozonation step. Fish exposed over 21 days to the ozonated STP effluent spawned significantly more eggs than fish exposed to the non-ozonated effluent and tap water. Vitellogenin induction (a biomarker for estrogenic pollutants) and a possible anxiety-related behavior were also observed in fish exposed to the ozonated STP effluent. Chemical analysis of the pre- and post ozonated STP effluent was used to screen for the presence of 105 pharmaceuticals, of which 24 could be detected. The average ozone removal efficiency of these pharmaceuticals was 77%.

The thesis work then focused on how ozonation would affect zebrafish embryotoxicity of three pharmaceuticals detected in the Knivsta STP effluent (i.e. carbamazepine, diclofenac, and oxazepam). While embryotoxicity of diclofenac was eliminated, exposure to oxazepam and carbamazepine induced embryotoxic responses following ozonation, suggesting formation of OBPs. The thesis therefore proceeded to investigate the toxicities of isolated carbamazepine OBPs. It was revealed that two carbamazepine OBPs, BQM and BQD, were the drivers of ozonated carbamazepine embryotoxicity. The thesis concludes that the formation of specific pharmaceutical OBPs explains adverse biological outcomes of effluent ozonation. The results add valuable information for the continuing efforts to improve STP effluent treatment.

Keywords: Zebrafish, Sewage treatment, Pharmaceuticals, Ozonation-by-products

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Effekter av ozonerade avloppseffluenter och läkemedel i sebrafisk (*Danio rerio*)

Sammanfattning

Vattenmiljön är slutdestinationen för många kemiska föroreningar av mänskligt ursprung, varav läkemedel är en viktig komponent. En väsentlig föroreningskälla är avloppsreningsverk vilka ofta saknar kapaciteten att effektivt avskilja läkemedelsrester. Flertalet studier har påvisat skadliga effekter av läkemedel i vattenlevande organismer. Nya avancerade avloppsreningsmotoder med potential att effektivt avskilja läkemedelsrester från avloppsvatten är under utredning. En av dessa metoder är ozonering av effluenten innan utsläpp i vattenmiljön. Ozonering av kemikalier, inklusive läkemedelsrester, kan dock potentiellt bilda toxiska ozonerings-restprodukter vilket gör det viktigt att studera de biologiska effekter som ozonering av avloppsvatten kan ha på vattenlevande organismer.

Denna doktorsavhandling hade som mål att utvärdera biologiska och kemiska effekter av ozonering av avloppseffluenter och läkemedel. I detta syfte användes en kombination av analytisk kemi och *in vivo* tester med sebrafisk (*Danio rerio*). Först undersöktes reproduktionsförmåga och beteende i vuxna sebrafiskar som exponerats för ozonerad avloppseffluent under 21 dagar vid Knivsta reningsverk. Kemisk analys av effluenten visade att ozonbehandlingen reducerade i snitt 77 % av koncentrationerna av 24 detekterade läkemedelssubstanser. Samtidigt hade fiskarna i den ozonerade effluenten bättre fekunditet än fiskar i den vanliga effluenten och kontrollgruppen. Vidare var genuttrycket av vitellogenin (en biomarkör för östrogena ämnen) inducerat och en förmodat stress-relaterad beteendeförändring uppmättes i dessa fiskar.

Avhandlingsarbetet fortsatte sedan undersöka ozonerat karbamazepin, diklofenak och oxazepam, läkemedel som förekom i Knivstaeffluenten. Ozonering reducerade diklofenaks embryotoxicitet men ökade toxiciteten av oxazepam och karbamazepin, vilket indikerade uppkomst av ozonerings-restprodukter. Vidare undersöktes därför vilka ozonerings-restprodukter som bidrog till den förstärkta embryotoxicititen av karbamazepin. Genom dessa försök konstaterades att två ozonerings-restprodukter, här kallade BQM och BQD, stod för ökningen av den embryotoxicitet som observerades efter ozonering av karbamazepin. Resultaten påvisar sammanfattningsvis att uppkomsten av specifika ozonerings-restprodukter förklarar ökad toxicitet av vissa läkemedel efter ozonering. Denna information är av vikt för det fortskridande arbetet att förbättra behandling av avloppsvatten med avseende på läkemedelsrester.

Nyckelord: Sebrafisk, avloppsrening, läkemedel, ozonerings-restprodukter

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Dedication

To Johanna, and the little one

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Pohl, J.***, Björlenius, B., Brodin, T., Carlsson, G., Fick, J., Larsson, D.G.J., Norrgren, L., Örn, S. (2018). Effects of ozonated sewage effluent on reproduction and behavioral endpoints in zebrafish (*Danio rerio*). *Aquatic Toxicology*, 200, pp. 93-101.

II **Pohl, J.***, Ahrens, L., Carlsson, G., Golovko, O., Norrgren L., Weiss, J., Örn, S. 2019. Embryotoxicity of ozonated diclofenac, carbamazepine, and oxazepam in zebrafish (*Danio rerio*). *Chemosphere*, 225, pp. 191-199.

III **Pohl, J.***, Golovko, A., Carlsson, G., Eriksson, J., Glynn, A., Örn, S., Weiss, J. 2020. Carbamazepine Ozonation Byproducts: Toxicity in Zebrafish (*Danio rerio*) Embryos and Chemical Stability. *Environmental Science & Technology*, accepted for publication.

* Corresponding author.

The contribution of Johannes Pohl (JP) to the papers included in this thesis was as follows:

I JP had the main responsibility of preparing and performing the zebrafish exposure study at Knivsta STP, as well as compiling literature and writing the manuscript with support from all co-authors. Journal correspondence was managed by JP.

II JP had the main responsibility of designing and executing the embryotoxicity experimental work of the manuscript. JP had the main responsibility for compiling literature and writing the manuscript with support from all co-authors. Journal correspondence was managed by JP.

III JP had the main responsibility of designing and executing the embryotoxicity experimental work of the manuscript. JP had the main responsibility for compiling literature and writing the manuscript with support from all co-authors. Journal correspondence was managed by JP.

Abbreviations

API	Active pharmaceutical ingredient
BOD_7	Biological oxygen demand 7 days
BQD	1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione
BQM	1-(2-benzaldehyde)-4-hydro-(1*H*,3*H*)-quinazoline-2-one
BVF	Department of Biomedical Sciences and Veterinary Public Health
CBZ	Carbamazepine
CBZ-EP	Carbamazepine 10,11-epoxide
DI-CBZ	10,11-Dihydrocarbamazepine
E2	17β-estradiol
EE_2	17α-ethinylestradiol
ERA	Environmental Risk Assessment
GAC	Granular activated carbon
Hpf	Hours post-fertilization
MBBR	Moving Bed Biofilm Reactor
NSAID	Nonsteroidal anti-inflammatory drug
OBP	Ozonation by-product
PAC	Powdered activated carbon
PE	Population equivalents
STP	Sewage treatment plant
SwAM	Swedish Agency for Marine and Water Management
WFD	Water Framework Directive

1 Introduction

1.1 Pharmaceutical residues in the aquatic environment

Chemical pollution originating from human activities can be found in virtually any environmental compartment on earth. The surface water environment is the major end destination for many organic micropollutants (i.e. substances quantified at ng−µg L^{-1}). Human and veterinary pharmaceuticals are among these so called 'emerging' contaminants commonly detected in surface water worldwide (Hughes *et al.* 2013; Luo *et al.* 2014; Beek *et al.* 2016). The therapeutic component of pharmaceuticals, the active pharmaceutical ingredient (API), is designed to interact with biological targets in the patient and to withstand transformation and elimination in the human body, before excretion. These characteristics have potentially problematic consequences in the environment as APIs can affect biological processes in a range of non-target aquatic organisms (Fent *et al.* 2006). Human drug target orthologues, meaning evolutionarily conserved drug target genes, are found in zebrafish (88%), *Daphnia pulex* (63%), and even in green algae *Chlamydomonas reinhardtii* (36%) (Gunnarsson *et al.* 2008).

A multitude of studies have provided evidence for hazardous effects in fish exposed to many different APIs. Some notable examples are intersex (i.e. hermaphroditism) caused by estrogenic compounds (Jobling *et al.* 1998; Woodling *et al.* 2006), altered behaviors in wild fish exposed to benzodiazepines (Brodin *et al.* 2013; Klaminder *et al.* 2019), and kidney lesions in fish exposed to diclofenac (Mehinto *et al.* 2010; Näslund *et al.* 2017). Pharmaceuticals may undergo biotransformation in the patient or environmental biota, in some cases increasing their toxicity to aquatic organisms. For instance, diclofenac can undergo methylation in the aquatic invertebrates *Gammarus pulex* and *Hyalella*

asteca, and in fish hepatocytes, leading to a 430-fold increase in acute toxicity of methylated diclofenac compared to the parent compound (Fu *et al.* 2020).

Pharmaceutical pollution has gained attention as a key global environmental issue requiring prioritization (Boxall *et al.* 2012). The European Union has acknowledged the issue by including diclofenac, 17β-estradiol (E2), 17α-ethinylestradiol (EE_2), and three macrolide antibiotics in the Water Framework Directive (WFD) list of candidate micropollutants that warrant environmental monitoring (European Union, 2015). It has been estimated that some pharmaceuticals, including diclofenac and EE_2, are present in several Swedish surface water environments at concentrations exceeding the water quality standards stipulated in the WFD depending on sampling location and time (Lindim *et al.* 2019).

Approximately 7500 pharmaceuticals are available on the global market today (The Royal Pharmaceutical Society, 2020). The European Medicine agency introduced environmental risk assessments (ERA) for all new pharmaceuticals developed from the year 2006. The back-catalog of 'legacy' pharmaceuticals (marketed before 2006) that are lacking environmental risk data are currently numbering 800 (Gunnarsson *et al.* 2019). An ERA of a new pharmaceutical is performed if the predicted environmental concentration (based on consumption data and dilution factors) exceeds 0.01 μg L^{-1}, if the molecule is lipophilic ($\log K_{ow} \geq 4.5$), or has suspected reproductive endocrine-disrupting properties (European Medicines Agency, 2006). An API can however not be barred from release to the market based on environmental concerns (European Medicines Agency, 2006).

Several issues regarding the ERA of pharmaceuticals have been raised. Ågestrand and co-workers have presented 10 recommendation to refine the ERA guideline, for instance inclusion of additional endpoints such as neurotoxicity and behavioral effects when assessing the environmental risks of pharmaceuticals, and to include the environmental aspect in the risk-benefit analysis of a new pharmaceutical before approval (Ågerstrand *et al.* 2015). Furthermore, as the assessments are based mainly on acute toxicity tests, there may be an underestimation of risk since aquatic organisms are often exposed chronically to pharmaceuticals (Ågerstrand *et al.* 2015). The actual internal concentrations of pharmaceuticals and their biotransformation products can be higher in the organisms than in their surrounding aquatic habitat, potentially underestimating environmental risk (Miller *et al.* 2018, 2019). Several pharmaceuticals have been shown to accumulate in fish blood plasma and other tissues (Fick *et al.* 2010; Zenker *et al.* 2014). The interaction effects of APIs may also increase the combined risk of pharmaceutical pollution, as the surface water environment generally contains a mixture of several pharmaceuticals (Backhaus,

2014). The environmental risks of mixtures is an area needing attention, highlighted in a recent statement by (Drakvik *et al.* 2020).

1.2 Sewage treatment plants – major sources of pharmaceutical pollution

A major contributor to pharmaceutical pollution is sewage treatment plant (STP) effluents which constantly flow into surface water. Raw sewage influent from industries, hospitals, and households generally contains a mixture of pharmaceuticals parent compounds and metabolites from a range of therapeutic classes (Luo *et al.* 2014). As an example, domestic and hospital sewage in Umeå, Sweden, contained 51 APIs at quantifiable concentrations, out of 105 analyzed (Lindberg *et al.* 2014). In Sweden, about 95% of sewage water is processed at STPs with chemical and biological treatment steps (Swedish Environmental Protection Agency, 2014). These sewage treatment steps are designed to remove phosphorus, nitrogen, and organic materials, in order to decrease bacterial concentrations and prevent eutrophication of the receiving water body. Many organic micropollutants, including pharmaceuticals, are however not readily removed by current 'conventional' treatment steps (Verlicchi *et al.* 2012). Further, de-conjugation of excreted conjugated pharmaceuticals may even increase concentrations of certain pharmaceuticals (e.g. antibiotics and carbamazepine) in sewage effluent as compared with sewage influent (Vieno *et al.* 2007; Gros *et al.* 2010).

Activated sewage sludge methods can increase microbial degradation of certain pharmaceuticals, like ibuprofen, naproxen and ketoprofen (Falås *et al.* 2012). Different types of filters can also aid in the removal of pharmaceuticals. Activated carbon filters with biofilm, e.g. granulated active carbon (GAC) or powder activated carbon (PAC), are able to physically adsorb and biologically degrade pharmaceuticals (Sbardella *et al.* 2018). PAC dosing in combination with moving bed biofilm reactors (MMBRs) can further improve the removal of pharmaceuticals (Cimbritz *et al.* 2019). About 25% of the produced sewage sludge is used as fertilizer on fields in Sweden (Statistics Sweden, 2012). Pharmaceuticals adsorbed in sewage sludge could potentially lead to terrestrial pharmaceutical pollution at agricultural sites where the sludge is used as fertilizer (Kümmerer, 2010; Ivanová *et al.* 2018). Studies on soil retention of pharmaceuticals have indicated persistence of several pharmaceuticals, including some psychoactive APIs (Magnér *et al.* 2016).

Pharmaceutical production sites are today largely located in Asia, where the situation regarding pharmaceutical pollution is considerably worse than in Sweden. Instead of pharmaceutical concentrations at the ng–µg L^{-1} range, some

sewage effluents and adjacent water bodies in Asia can have concentrations exceeding mg L^{-1} levels (Larsson 2014). As an example, an STP located in a heavily industrialized part of Hyderabad, India, produced an effluent containing 1 000 000–fold higher concentrations of the antimicrobial ciprofloxacin compared with average content in effluents from Swedish STPs (Larsson *et al.* 2007). Ecotoxicity screenings of water collected at these locations indicated sublethal effects in amphibian tadpoles (Carlsson *et al.* 2009) and fish (Gunnarsson *et al.* 2009a) already at 0.2% dilution. Highly urbanized areas with dense populations can also produce STP effluents with elevated pharmaceutical concentrations, potentially leading to high pollution loads locally (Beek *et al.* 2016). Interestingly, chemical screening of sewage effluents can reveal consumption patterns of illicit narcotic drugs; a study on STP effluent water collected during seven music festivals in the Czech and Slovak Republics could associate different classes of drugs to specific music genres, Ecstasy concentrations peaked for example during an electronic dance festival (Mackuľak *et al.* 2019). These types of psychotropic drugs can also cause distinct behavioral effects in fish (Neelkantan *et al.* 2013).

The Swedish Environmental Protection Agency, together with some of Sweden's county administrative boards, has commissioned screening studies of pharmaceutical residues in sewage influent, effluents, sludge, recipient waters, and fish tissues. These screenings have reported high concentrations of pharmaceuticals in sewage effluents and sewage sludge, and the presence of pharmaceuticals and estrogens in wild fish bile (Fick *et al.* 2011, 2014). The concentrations of some pharmaceuticals measured in surface water in these screenings exceeded the predicted pharmacological effect concentrations (Fick *et al.* 2010).

1.3 Ozonation – a method to improve pharmaceutical removal in sewage effluents

The presence of micropollutants in STP effluents is prompting mitigation measures such as improved sewage treatment (Eggen *et al.* 2014). One potential treatment technology is ozonation. Ozonation has been used for water treatment since the 19th century in both the United States (McGuire, 2006) and Europe (Paulouë & Langlais 1999). The fate of the ozonated pharmaceuticals and other organic molecules present in the STP effluent is dependent on a multitude of factors including ozone dose and composition of the effluent (Antoniou *et al.* 2013; Zucker *et al.* 2015). Ozone directly targets and oxidases electron-rich moieties in the ozonated molecule. Ozonation of sewage effluents, which generally contain high concentrations of dissolved organic matter, produce

hydroxide-radicals that proceed to non-selectively degrade organic molecules, such as pharmaceuticals (Buffle *et al.* 2006). The ozonation process also forms many potentially toxic ozonation by-products (OBPs), e.g. aldehydes, ketones, and carboxylic acids (Hammes *et al.* 2006; Wert *et al.* 2007), bromate (von Gunten & Hoigne 1994), and N,N-dimethylsulfamide (Schmidt & Brauch 2008).

Experimental studies have reported efficient pharmaceutical elimination of e.g. diclofenac and carbamazepine by ozonation (Huber *et al.* 2005; Buffle *et al.* 2006). Investigations on full-scale ozonation steps integrated at STP have also shown potential in drastically improving pharmaceutical removal (Baresel *et al.* 2016; Bourgin *et al.* 2018). Pharmaceutical ozonation will not necessarily lead to complete mineralization, but rather formation of a multitude of OBPs. While novel chemical analysis methodologies have increased the knowledge of OBPs formation and reaction kinetics, there are data gaps regarding the toxicities of these kinds of compounds (Cwiertny *et al.* 2014; von Gunten, 2018). The present thesis work, therefore, focused on this aspect in Papers II and III.

1.4 Knivsta STP full-scale ozonation pilot plant

The Swedish government proposition 2013/14:1 allocated 32 million Swedish crowns to the Swedish Agency for Marine and Water Management (SwAM) in order to stimulate research and development of advanced sewage treatment for removal of pharmaceuticals and other micropollutants. SwAM proceeded to grant eight projects that ran between 2014 and 2017 (see Cimbritz & Mattsson (2018) for project summaries). One of the funded projects, 'Evaluation of Advanced Treatment in Full-scale', was located at Knivsta municipal STP and was the first operational full-scale effluent ozonation plant constructed in Sweden in late 2014 (Björlenius 2018). The SwAM-funded project included chemical analysis of pharmaceuticals in pre- and post-ozonated effluent, ecological status in the recipient stream (Knivstaån) before and after the ozonation campaign, impacts on pathogenic virus and bacteria by ozonation of the effluent, and biological effects in fish (part of the thesis work, Paper I).

1.5 The zebrafish (*Danio rerio*) as a model species for aquatic ecotoxicology

The zebrafish is a small (adult size 3-5 cm) freshwater cyprinid fish native to the Ganges and Brahmaputra river basins on the Indian subcontinent (Spence *et al.* 2008). The natural habitats of the zebrafish are slow-moving streams and floodplains, where the fish spawn just before the rainy monsoon season (Spence *et al.* 2008). The zebrafish has become a popular model species in a wide range

of scientific fields, from the behavioral and pharmacological sciences to environmental toxicology (Dai *et al.* 2014). This owes to the fact that zebrafish readily reproduce under controlled laboratory settings. Zebrafish embryos have been used for more than 60 years within the context of teratogenic (i.e. developmental disruption) effects of chemicals (Jones & Huffman 1957). The eggs are completely transparent which allows inspection of embryo morphology and bodily functions (i.e. heart rate) by stereomicroscope.

The zebrafish embryo has become an important model for toxicity testing, as it is generally not considered to be an animal model up until the point of feeding, thus not requiring ethical permission (Scholz *et al.* 2008; Strähle *et al.* 2012; Braunbeck *et al.* 2015). The embryo-larvae nevertheless represents an *in vivo* test system enabling metabolic, toxicodynamic and toxicokinetic assessment of xenobiotics (Kühnert *et al.* 2013; Vogs *et al.* 2019). The embryo normally develops to sexual maturity in a matter of months, allowing for multigenerational studies, including exploration of transgenerational epigenetic mechanisms (Blanc *et al.* 2019). The whole zebrafish genome has been sequenced and extensively annotated, and has a high resemblance to higher vertebrate models, including humans (Howe *et al.* 2013). The robust behavioral repertoire of both adult and larval zebrafish has also made them utilized in studies of neuroactive pharmaceuticals (Kalueff *et al.* 2016). The aforementioned characteristics promoted the use of zebrafish in the studies presented in this thesis.

2 Aims of the thesis

The overarching aim of this thesis was to explore the outcomes of ozonation treatment of STP effluents with a special focus on pharmaceuticals, by combining chemical analysis with zebrafish *in vivo* testing. The thesis work was divided into three parts. Firstly, a Swedish STP (Knivsta municipal STP) with full-scale ozonation treatment was evaluated for biological effects on reproduction and behavior in adult zebrafish (Paper I). Thereafter, selected pharmaceuticals (i.e. diclofenac, carbamazepine, and oxazepam) detected in the Knivsta STP effluent were treated in a laboratory-scale ozonation reactor and tested for zebrafish embryotoxicity (Paper II). As a direct follow-up to Paper II, where it was discovered that carbamazepine embryotoxicity increased following ozonation, carbamazepine OBPs were isolated from the ozonated solution and tested individually and in a mixture in zebrafish embryos (Paper III).

The thesis encompassed the following research questions:

- How much will the implementation of a full-scale ozonation treatment step at Knivsta STP improve pharmaceutical removal efficiency?
- Will the ozonation of the Knivsta STP effluent affect biological responses in exposed adult zebrafish?
- How may ozonation modulate zebrafish embryotoxicity of the three common pharmaceutical micropollutants carbamazepine, diclofenac, and oxazepam?
- Which OBPs are responsible for induced carbamazepine embryotoxicity following ozonation?
- Are carbamazepine OBPs stable in water solution during storage for two weeks at laboratory conditions?

3 Materials and methods

The following sections briefly summarize the study designs and provide rationales for endpoint selection of the different studies within the thesis. All experimentation on adult fish was approved beforehand by the Animal Ethics Committee in Gothenburg (DNR79-2014).

3.1 Study designs

The thesis work centered on two experimental settings; a full-scale ozonation pilot plant at Knivsta municipal STP, and a bench-scale laboratory ozonation reactor (Figure 1).

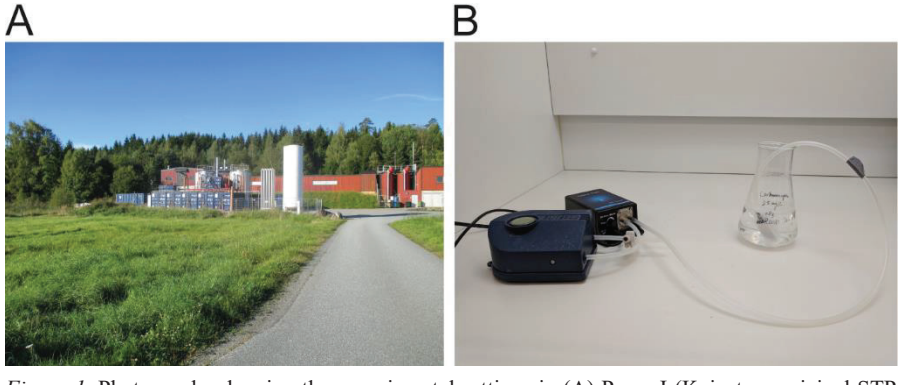


Figure 1. Photographs showing the experimental settings in (A) Paper I (Knivsta municipal STP with full-scale ozonation plant, photographer: Berndt Björlenius) and (B) Papers II and III (Bench-scale experimental ozonation reactor, photographer: Johannes Pohl).

3.1.1 Knivsta sewage treatment plant (Paper I)

The first part of the thesis work (Paper I) aimed to evaluate *in vivo* endocrine, reproductive, and behavioral responses in zebrafish exposed to the ozonated Knivsta STP effluent at a full-scale experimental ozonation treatment facility. The ozonation removal efficiency of 105 pharmaceuticals in the treated effluent was also examined. The on-site study at Knivsta STP was conducted in November 2015. At the time Knivsta municipal STP had a treatment capacity of 13 000 population equivalents (PE) at 300 m^3 sewage per hour, with approximately 11 000 connected persons in 2015 (Roslagsvatten, 2016). The mean volume treated sewage in 2015 was 3708 m^3 per day, and a maximum flow of 8600 m^3 per day (Roslagsvatten, 2016). Mostly household spill water was treated at the STP, no major industries were connected at the time of the study (Roslagsvatten, 2016).

Knivsta STP had five major treatment steps at the time of the ozonation project: pretreatment (3 mm bar distance screen), primary sedimentation with chemical precipitation, biological treatment (active sludge pools and nitrification reactors with aggregate), secondary sedimentation, and post-chemical precipitation (iron chloride PIX-111). The mean hydraulic retention time in Knivsta STP during the time of the study was 38 hours, producing an effluent with < 3 mg L^{-1} biological oxygen demand 7 days (BOD_7), 9 mg L^{-1} total nitrogen, 0.11 mg L^{-1} total phosphorus, and 0.09 mg L^{-1} ammonia nitrogen. In 2015, Knivsta STP effluent mean concentrations of cadmium, copper, and zink were 0.021, 1.6, and 15.7 µg L^{-1}, respectively (Roslagsvatten, 2016). After treatment, the effluent was released into a constructed wetland polishing pond from where it discharged into the recipient stream, Knivstån.

The ozonation step (7 mg L^{-1} ozone dose) was connected after the conventional treatment, which allowed parallel studies on ozonated- and nonozonated effluent (see detailed description of the ozonation plant in the materials and methods sections in Östman *et al*. (2019) and Paper I). Contact filters filled with expanded clay aggregates were installed after ozonation for reducing any remnant ozone. A mobile laboratory (isolated 10 ft ISO-container) containing aquariums (n = 3 per treatment, n = 10 fish, ~5 males and 5 females, per aquaria) for the fish studies was installed next to the ozonation plant. Water from effluent (pH: 7 ± 0.05, nitrate: 84 ± 4 mg L^{-1}) ozonated effluent (pH: 7.5 ± 0.02, nitrate: 101 ± 5 mg L^{-1}), and carbon-filtered municipal tap water (pH: 7.5 ± 0.09, nitrate: < limit of measurement) was continuously pumped into 60 L polyethylene plastic barrels, warmed to 25 °C by immersion heaters, and distributed into aerated aquaria at a flow of 10 mL min^{-1} (Figure 2).

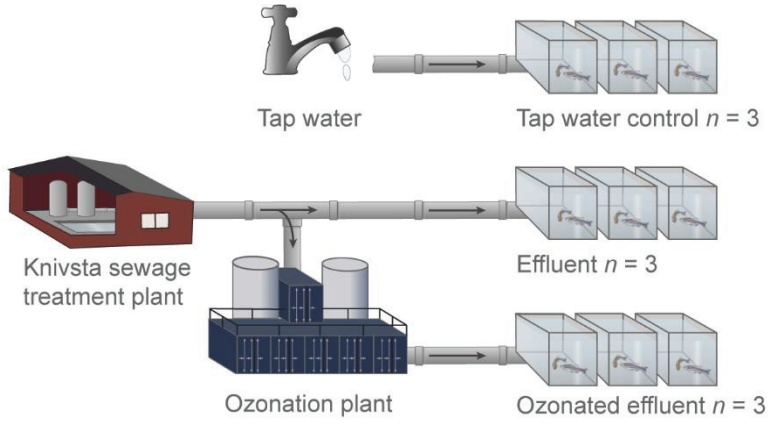
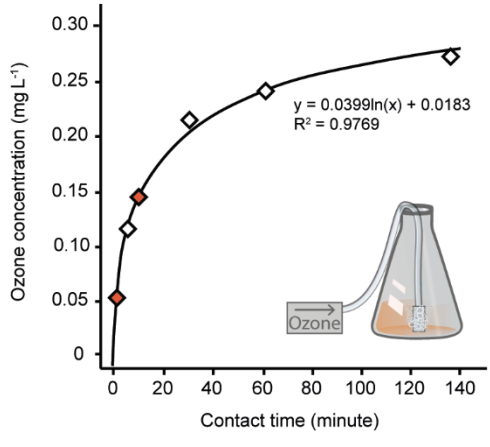


Figure 2. Experimental setting used in Paper I. Carbon-filtered water from the municipal tap water system, treated sewage effluent from the Knivsta STP, and ozonated effluent was continuously pumped into the aquariums (n = 3 per treatment) on-site.

3.1.2 Bench-scale laboratory ozone reactor (Papers II and III)

A



B

Study	Contact time (min)	Peak [O_3] (mg L^{-1})*
Paper II	1	0.053
	10	0.147
Paper III	10	0.29

* Measured at end of ozonation

Figure 3. (A) Dissolved ozone concentration (mg L^{-1}) in the ozonation reactor (0.25 L carbon filtered tap water at 25 °C) measured at 1, 5, 10, 30, 60, and 135 min after initiation of the ozonation process (determined in Study II). (B) Table of ozone contact times and peak ozone concentrations in Papers II and III.

An experimental bench-scale ozonation reactor was set up for the purpose to ozonate pharmaceuticals (Papers II and III). An ozone generator (Aqua Medic GMBH, Germany) supplying 100 mg O_3 per hour was used to ozonate test

solutions (v = 0.25 L, 25 °C) by way of diffusion stone in Erlenmeyer flasks. All pharmaceuticals were mixed in carbon-filtered tap water which also constituted the negative control. A preliminary test of ozone concentrations in the reactor during continuous ozonation was performed to evaluate ozonation efficiency before commencing the studies presented in Paper II (Figure 3). Ozone concentrations were measured using the LCK310 Ozone cuvette test (0.05−2 mg L^{-1} O_3 measurement range) in a DR 3900™ spectrophotometer (Hach, United States). The set up was localized to a fume hood in order to dissipate residual ozone gas.

From the preliminary test, two ozone dosages were selected for the subsequent treatment of the tested pharmaceuticals in Paper II (Figure 4); 1 min ozonation (peak dissolved ozone concentration of 0.053 mg L^{-1}), and 10 min ozonation (0.147 mg L^{-1}). The rationale for choosing these two dosages was mainly due to practical feasibility. In order to complete ozonation and initiate the embryotoxicity experiments during the same morning, additional or longer ozonation dosages would not have been possible.

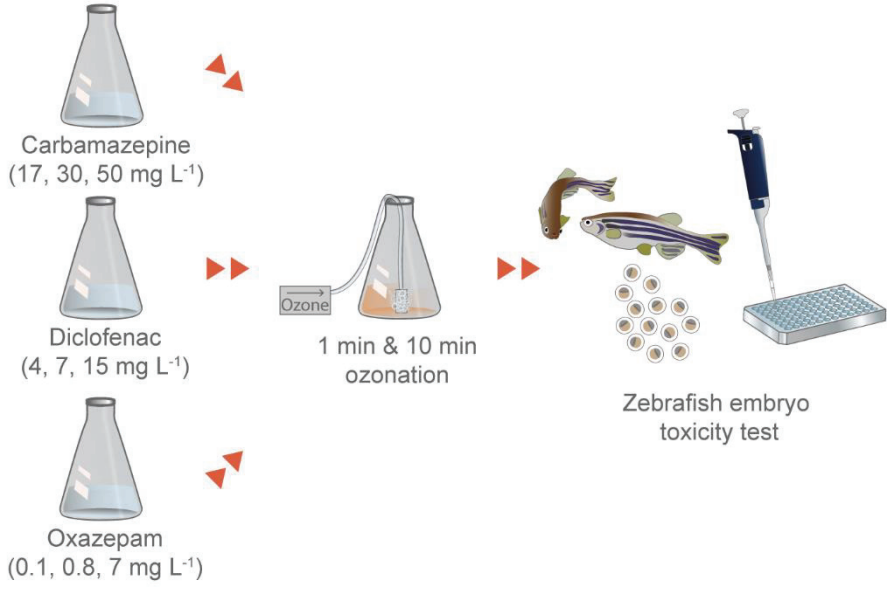


Figure 4. Experimental setting used in Paper II. Three concentrations of each pharmaceutical were selected by preliminary toxicity screening and ozonated for 1 and 10 minutes.

The last part of the thesis work (Paper III) aimed to further investigate individual carbamazepine OBPs isolated from ozonated solution (Figure 5). Zebrafish embryo assays were used to test the individual and mixture toxicity of the four carbamazepine OBPs 10,11-epoxide (CBZ-EP), 10,11-Dihydrocarbamazepine

(DI-CBZ), 1-(2-benzaldehyde)-4-hydro-(1*H*,3*H*)-quinazoline-2-one (BQM), and 1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione (BQD).

The test concentrations were selected based on quantified concentrations in ozonated CBZ. Three concentration levels were tested, corresponding with approximately 0.5×, 1×, and 2× the concentrations of each respective OBP. Since BQM and BQD were not commercially available they had to be extracted from ozonated carbamazepine. The isolated compounds were eluted in methanol (evaporated before addition of carbon-filtered tap water) and tested for embryotoxicity in the zebrafish embryo assay along with CBZ-EP and DI-CBZ which were prepared from analytical-grade powder in carbon-filtered tap water.

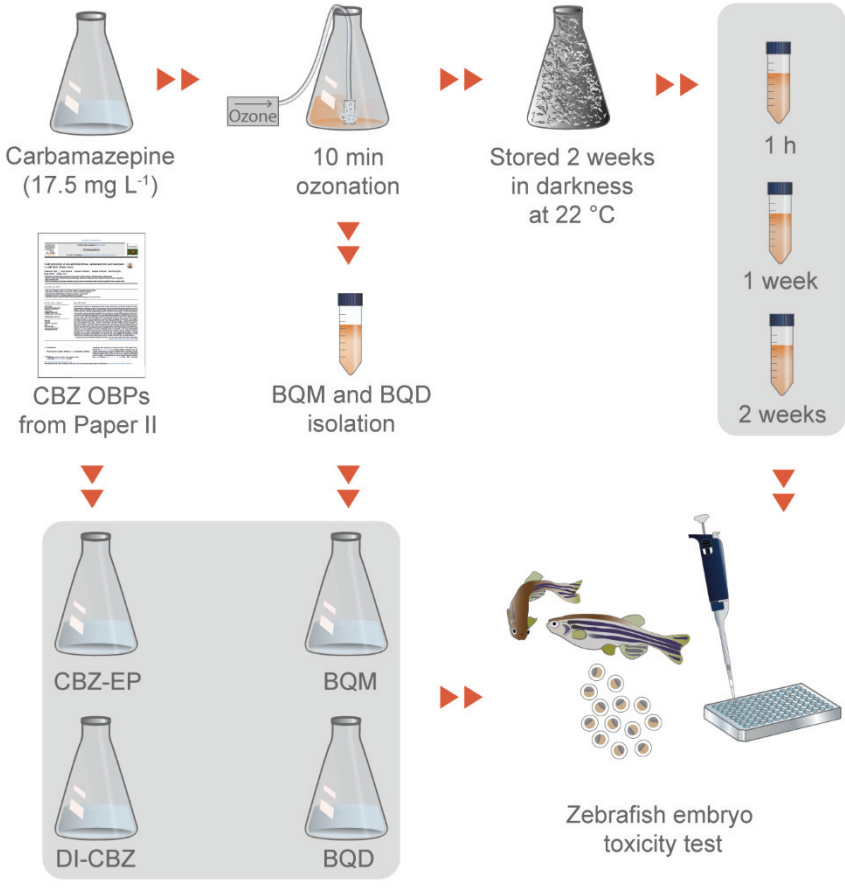


Figure 5. Experimental setting used in Paper III. Four carbamazepine OBPs were tested in zebrafish embryo toxicity tests individually and in a mixture. The ozonated carbamazepine solution was stored in the dark at 22 °C for two weeks and repeatedly tested for embryotoxicity and OBP concentration after 1h, 1 week, and 2 week storage.

3.2 Adult zebrafish biological endpoints (Paper I)

3.2.1 Reproduction

Adult zebrafish were exposed to the Knivsta STP effluent pre- and postozonation for 21 days and tested for fecundity and behavioral effects (Figure 6). The reproductive status was evaluated by measuring fecundity (i.e. the number of spawned fertilized eggs per female per day). This method was selected as it has been utilized in earlier fecundity studies in zebrafish (Lister *et al.* 2009). Spawned eggs were collected from each aquarium after each 'spawning trail', counted, and assessed for fertilization success (i.e. if the egg had progressed beyond the 4-cell stage) once per day during the last 7 days of the 21-day exposure. The spawning trails began by moving the fish into metal mesh cages (5 mm mesh size) at 09:00 (when the aquarium lights were turned on) and allowing them 30 min to spawn. This method tend to stimulate zebrafish spawning. Each aquarium population, with five putative males and five putative females, was considered a replicate.

At the termination of the 21 day exposure exposure, the fish were euthanized and prepared for histological sectioning of gonadal tissue. Fish were fixed in phosphate-buffered formaldehyde, embedded in paraffin blocks, and dehydrated before sectioned and stained with haematoxylin-eosin. Prepared sections were analyzed under stereo microscope. To confirm fecundity effects, the histological status of the ovaries (i.e. the proportion of primary, cortical alveolar, and mature vitellogenic oocytes) was investigated in female fish. Male gonadal tissues were assessed for semen content in seminiferous tubuli, and signs of intersex was also examined.

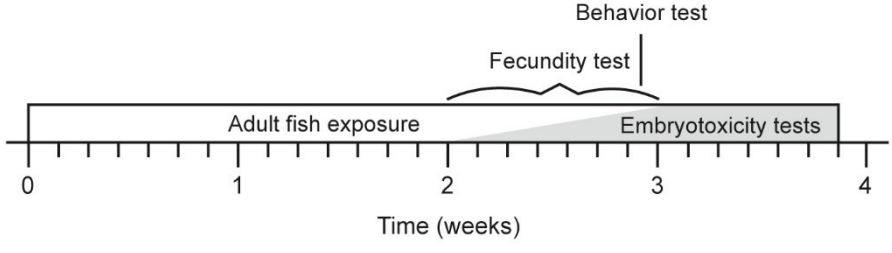


Figure 6. Experimental timeline for the zebrafish study at Knivsta STP (Paper I). Fish were exposed over three weeks, where the last week consisted of a fecundity test, and the last day consisted of a behavior test. Embryos were collected from the first successful spawning in each aquarium and tested for embryotoxicity in a 6 day embryo toxicity test.

3.2.2 Gene expression analysis

Zebrafish hepatic tissue was homogenized using a Precellys Evolution (Bertin instruments, France) system equipped 190 with liquid nitrogen cooling. RNA was then extracted using a QiaSymphony instrument with standard reagents (Qiagen, Germany). The RNA integrity and concentration were measured in a 2100 Bioanalyzer system (Agilent Technologies, USA) to ensure sample template quality. Quantitative polymerase chain reaction (qPCR) was performed using a Rotor-Gene 3000 instrument (Corbett Research, Australia) with the one-step KAPA SYBR® FAST one-step qPCR kit, according to the manufacturers recommended protocol. The primer sequences were synthesized, evaluated and the reaction products were sequenced to ensure product specificity. Relative mRNA expression was calculated by the method described by Livak and Schmittgen (2001). Three genes were selected for gene expression analysis: *cyp1a1* (dioxin responsive gene), *ERa*, and *vtg2* (xenoestrogenic responsive genes). These genes have been used as biomarkers in earlier studies on ozonated sewage effluents (Gunnarsson *et al.* 2009b; Beijer *et al.* 2017).

3.2.3 Adult behavior

The adult zebrafish behavior was tested in a novel tank test, meaning that fish were transferred individually together with 0.5 L aquarium water to unfamiliar non-transparent glass beakers (12 cm in diameter). After placement in the glass beakers, fish were filmed by an above-mounted video camera over 10 min. Locomotor activity was then tracked using Zebralab software (ViewPoint, France). A stressed zebrafish will freeze when introduced to a novel environment, before starting to investigate its surroundings (Demin *et al.* 2020). This metric for anxiety-like response was a simplified version of more refined methods, including time spent bottom-dwelling and response to visual or olfactory distress cues (Kalueff *et al.* 2013).

3.3 Zebrafish embryo-larvae biological endpoints (Papers I-III)

Fertilized zebrafish embryos were exposed individually in Parafilm M (Bemis Company, United States) 96-well plates for 144 hours post- fertilization (hpf). The method has been extensively used and characterized at the Department of Biomedical Sciences and Veterinary Public Health (BVF) aquatic facility were all studies took place (Carlsson, 2007; Carlsson *et al.* 2011; Carlsson & Norrgren 2014). The zebrafish embryotoxicity assays were performed under controlled ambient conditions (12:12 h light cycle, 26 ± 1 °C air temperature).

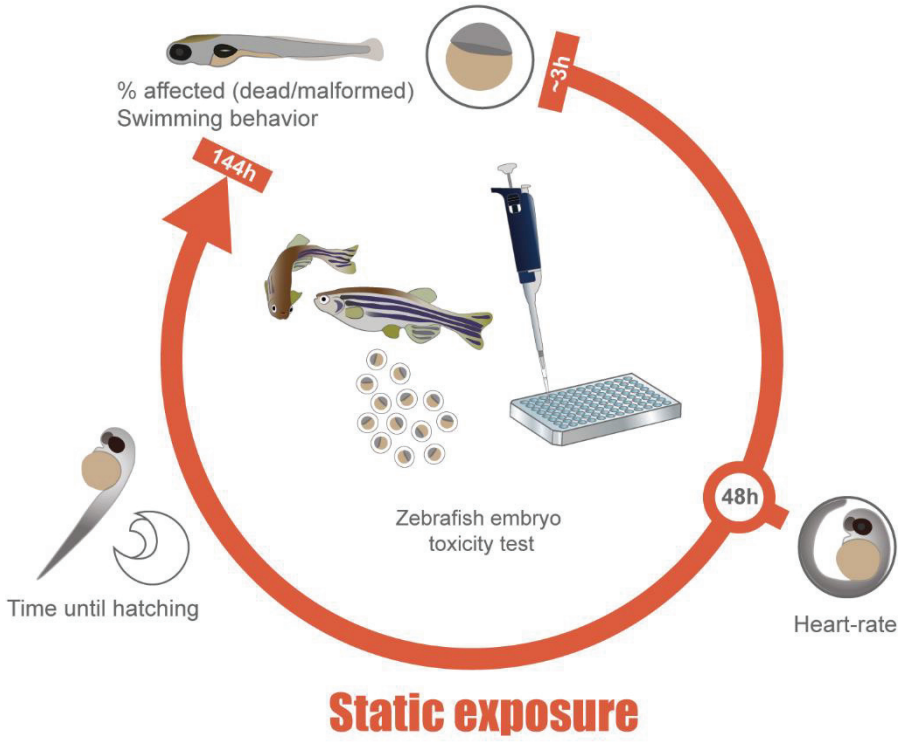


Figure 7. Overview of the zebrafish embryotoxicity assay experimental timeline used in the thesis work.

All exposures were static (i.e. exposure solutions were not changed during the tests). Heart rate at 48 hpf, time until hatching (h), swimming activity during alternating light/dark periods, and proportion of affected (dead and malformed) embryos at 144 hpf were the recorded endpoints (Figure 7). The embryos were assessed under stereo microscope for observable morphological deformities such as pericardial edema, yolk sac edema, and scoliosis.

Heart rate was recorded while minimizing confounding factors. Each plate was measured over the course of about 45 minutes, under which time the measured air temperature did not fluctuate. The Parafilm M cover on the plates was not removed during heart rate measurements (inverted microscope was used in order to observe the plates from below). An external LED-light was used to illuminate the embryos at equal intensity to avoid temperature increase. The heart rate (heartbeats per minute, bpm) was then calculated by the formula

$$30 \times \frac{60}{time\ for\ 30\ heartbeats\ (s)}$$

The hatching time was recorded by photographing (Canon EOS 500D) the 96-well plate(s) each hour between ~48 hpf and 144 hpf. A timed LED lighting system provided light during photography (switched on for 3 seconds) in the dark hours. The image files were manually inspected to deduce which hpf each larva hatched.

The zebrafish larvae behavior in response to shifts in light intensity was measured using the Zebrabox tracking system and software (Viewpoint, France). The larvae locomotor activity has been recommended as a rapid and valuable test for neurodevelopmental effects in fish larvae (MacPhail *et al.* 2009). The behavioral phenotype (hypoactive or hyperactive) of each individual larvae was assessed by tracking the total locomotor activity (distance moved) during light and dark conditions. Only alive larvae without any observable malformations were included in order not to confound subsequent data analysis. The data was parsed, analyzed, and visualized using custom R software scripts (Pohl 2019).

The embryotoxicity tests performed in Paper I using embryos and water from Knivsta STP were simplified as to only record incidence of affected (dead/malformed) embryo-larvae and locomotor activity during alternating light and dark periods. This method was used due to time and logistical constraints. Eggs from the first successful spawning from each aquarium were selected for assessment, and placed in 96-well plates together with treatment water from the respective aquarium. The exposure itself took place at the BVF aquatic facilities.

Before commencing the ozonation tests in Paper II, concentration-response screenings for each of the selected pharmaceuticals (carbamazepine, diclofenac, and oxazepam) were performed. Three exposure concentrations (with effects ranging from none/weak in the lowest concentration to strong in the highest concentration), were identified and subsequently ozonated to evaluate how ozone would modulate their embryotoxicity.

3.4 Chemical analysis

Details on chemical analysis methodology and the OBP isolation protocol are found in the respective Materials & Method sections of Papers I-III.

3.5 Statistics

The R software (R Core Team, 2019), Rstudio interface (RStudio Team, 2019) and a collection of third-party packages (detailed in respective paper) were used for all statistical modeling, analysis, and plotting. Statistical methodology is elaborated upon in each respective paper.

4 Results and discussion

The results from Papers I-III are presented and discussed in the following sections. Figure 8 summarizes the most important results gathered during the thesis work.

4.1 Full-scale ozonation at Knivsta STP (Paper I)

4.1.1 Presence of pharmaceuticals in effluent and ozonation removal efficiency

Chemical analysis was performed to assess the ozone removal efficiencies of pharmaceuticals at Knivsta STP (Paper I). Chemical analysis of the Knivsta STP effluent showed that 24 out of 105 target pharmaceuticals could be detected. The sum concentration of quantified pharmaceuticals in the effluent was 6392 ng L^{-1}. After ozonation, 11 pharmaceuticals remained with a sum concentration of 1421 ng L^{-1} (77% average ozone removal efficiency on mass). Oxazepam was among the least efficiently removed APIs (42%) by the ozone treatment. Most other detected pharmaceuticals were removed to a higher extent, with the examples of diclofenac (99%) and carbamazepine (97%). A complete table containing ozone removal efficiencies of screened pharmaceuticals can be found in Table 1 in Paper I. The ozonation removal efficiency of the quantified pharmaceuticals reported in Paper I was in line, albeit a little bit lower compared with earlier studies on sewage ozonation (Lavén *et al.* 2009; Beijer *et al.* 2017). This may have been due to the ozone dosage not being adjusted for fluctuations in total organic carbon content in the effluent, leading to a lower ozone concentrations due to ozone scavenging. Subsequent biological treatment of sewage effluents resulting in lower levels of dissolved organic carbon has been shown to be important for efficient ozonation removal of pharmaceuticals and other micropollutants (Ekblad *et al.* 2019).

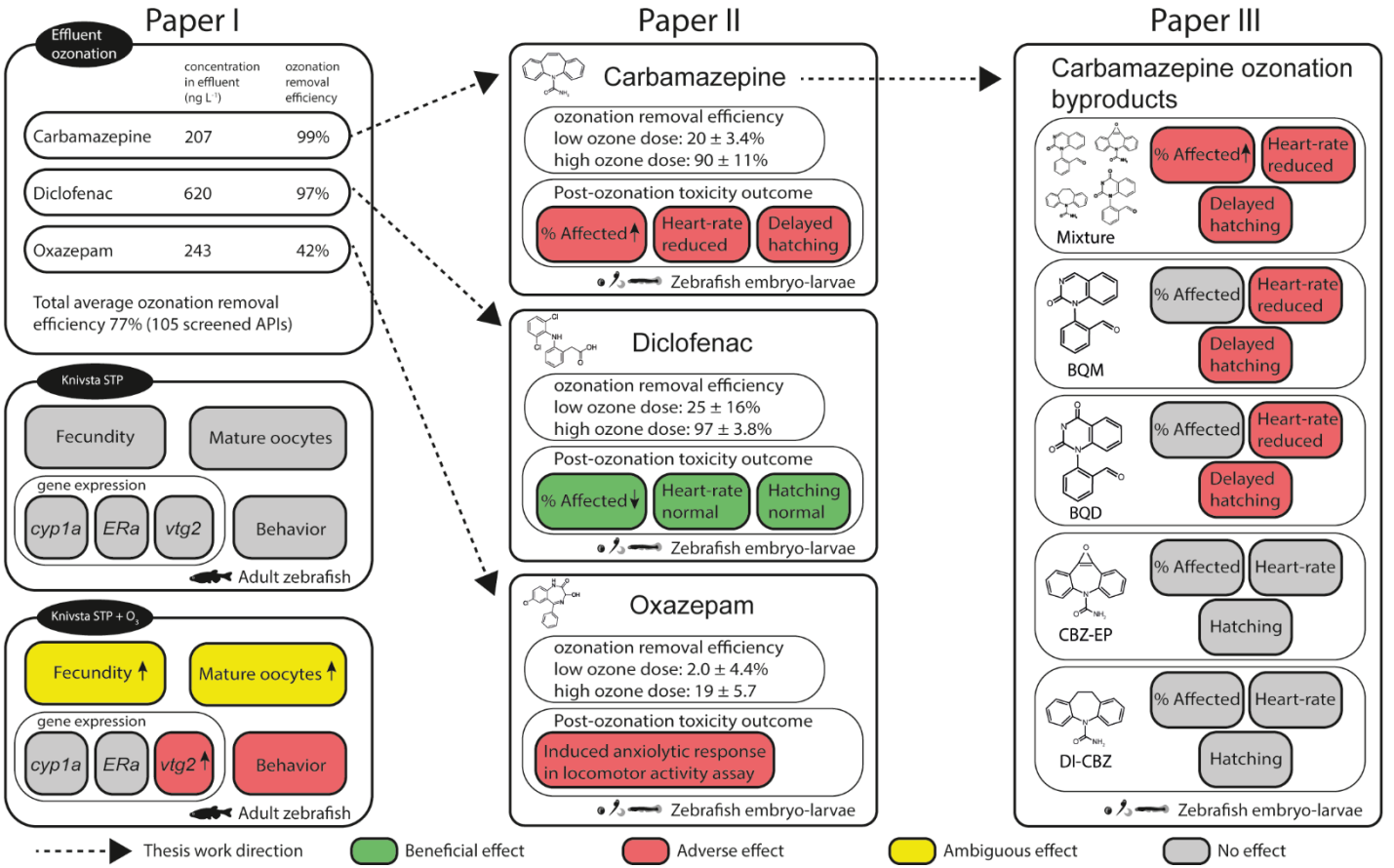
During the operation of the Knivsta full-scale ozonation plant, a pilot treatment system containing several additional sewage treatment technologies (e.g. GAC) was evaluated with the same effluent and ozonated effluent line (Östman *et al.* 2019). It was observed that the GAC filter was more efficient than ozonation for the removal of a list of antibiotic pharmaceuticals in the effluent (Östman *et al.* 2019). GAC filters could also work particularly well for the removal of OBPs (Schollée *et al.* 2018). In general, the addition of a post-ozonation filter (e.g. sand filter or GAC) seem to efficiently reduce effluent toxicity due to OBP abatement (Prasse *et al.* 2015; Völker *et al.* 2019). The chemical analysis of the ozonated Knivsta STP effluent did however not screen for the formation of any transformation-products or pharmaceutical OBPs (the OBP route of investigation was continued in Papers II and III).

Non-target chemical analysis is promising for detecting OBPs, but should be coupled with ecotoxicologal evaluations of ozonated effluent to limit the amount of data to be analyzed in the search for key toxicants (Nürenberg *et al.* 2019). *In vitro* effect-based studies have deduced that only 1-3% of toxicity following sewage effluent ozonation could be explained by the list of analyzed micropollutants (predominately pharmaceuticals), indicating significant toxic potencies of e.g. OBPs (Escher *et al.* 2013; Tang *et al.* 2013). There is a wealth of scientific literature reporting diverging biological outcomes in fish exposed to ozonated effluents, both beneficial and adverse (Table 1). The biological outcomes of ozonated Knivsta STP effluent in exposed zebrafish fell into two categories: ambiguous and potentially adverse (Figure 8).

4.1.2 Zebrafish fecundity, gonadal histology, and estrogen-associated gene expression

There were no mortalities observed among the exposed fish during exposure to the Knivsta STP effluent pre- or postozonation. The mean nitrate concentrations measured in the effluent water (84 ± 4 mg L^{-1}) and ozonated effluent (101 ± 5 mg L^{-1}) were not so high as to be considered toxic to zebrafish (Learmonth & Carvalho 2015).

Figure 8 (next page). Schematic flow-chart of the thesis work highlighting important findings. The outcomes from the zebrafish exposure studies have been color-coded according to the subjectively interpreted nature of the effect (i.e. beneficial, adverse, and ambiguous) or no effect.


Paper I
Effluent ozonation
concentration in effluent (ng L-1)
ozonation removal efficiency
Carbamazepine 207 99%
Diclofenac 620 97%
Oxazepam 243 42%
Total average ozonation removal efficiency 77% (105 screened APIs)
Knivsta STP
Fecundity
Mature oocytes
gene expression
cyp1a
ERa
vtg2
Behavior
Adult zebrafish
Knivsta STP + O3
Fecundity ↑
Mature oocytes ↑
gene expression
cyp1a
ERa
vtg2 ↑
Behavior
Adult zebrafish
Paper II
Carbamazepine
ozonation removal efficiency
low ozone dose: 20 ± 3.4%
high ozone dose: 90 ± 11%
Post-ozonation toxicity outcome
% Affected ↑
Heart-rate reduced
Delayed hatching
Zebrafish embryo-larvae
Diclofenac
ozonation removal efficiency
low ozone dose: 25 ± 16%
high ozone dose: 97 ± 3.8%
Post-ozonation toxicity outcome
% Affected ↓
Heart-rate normal
Hatching normal
Zebrafish embryo-larvae
Oxazepam
ozonation removal efficiency
low ozone dose: 2.0 ± 4.4%
high ozone dose: 19 ± 5.7
Post-ozonation toxicity outcome
Induced anxiolytic response in locomotor activity assay
Zebrafish embryo-larvae
Paper III
Carbamazepine ozonation byproducts
Mixture
% Affected ↑
Heart-rate reduced
Delayed hatching
BQM
% Affected
Heart-rate reduced
Delayed hatching
BQD
% Affected
Heart-rate reduced
Delayed hatching
CBZ-EP
% Affected
Heart-rate
Hatching
DI-CBZ
% Affected
Heart-rate
Hatching
Zebrafish embryo-larvae
Thesis work direction
Beneficial effect
Adverse effect
Ambiguous effect
No effect

Reproductive success was significantly higher in fish exposed to the ozonated STP effluent compared to the tap water control and conventional STP effluent. The average number of spawned fertilized eggs per female was 77.5 ± 7.5 in the ozonated effluent treatment, approximately double compared with the STP effluent (38.5 ± 1.9) and tap water control (34.4 ± 13.8) treatment groups (ANOVA $p = 0.0021$).

Table 1. *Non-exhaustive selection of peer-reviewed articles reporting effects of ozonated effluents in fish, showcasing different outcomes due to ozonation.*

Species	Life stage	Endpoint	Outcome following ozonation	Interpretation of effect	Reference
Japanese medaka (*Oryzias latipes)*	Embryo-larvae	Hatching success	Decreased	Adverse	Cao *et al*. (2009)
		Mortality	Increased	Adverse	
		Deformity	Increased	Adverse	
		Hatching success	Decreased	Adverse	Yan *et al*. (2014)
		Mortality	Increased	Adverse	
		Deformity	Increased	Adverse	
Rainbow trout (*Oncorhynchus mykiss)*	Embryo-larvae	Whole-body vitellogenin	Reduced	Beneficial	Stalter *et al*. (2010)
		Mortality	Increased	Adverse	
		Hatching success	Returned to normal	Beneficial	Margot *et al*. (2013)
		Mortality	Returned to normal	Beneficial	
Zebrafish (*Danio rerio*)	Adult	Reproduction	Increased	Ambiguous	Pohl *et al*. (2018)
		Behavior	Induced stress	Adverse	
		Mortality	Increased	Adverse	da Costa *et al*. (2014)
	Embryo-larvae	Hatching success	Returned to normal	Beneficial	Lundström *et al*. (2010)

Histological sections of female fish ovaries revealed that female fish exposed to the ozonated sewage effluent contained a two-fold higher proportion vitellogenic (i.e. mature) oocytes compared with the tap water control group ($p = 0.0424$). The histological assessment thus corroborated the results on egg production output in the fish. Further, histological evaluation of the male gonads in Paper I revealed no signs of intersex in any individual, and all males had normal seminiferous tubules with similar quantities of spermatozoa.

Whether the induced reproductive output should be considered beneficial or adverse for fish fitness in the environment is highly speculative, and may therefore be considered an ambiguous outcome. The increased fecundity could potentially be associated with the increased estrogenicity of the post-ozonated effluent, as observed in the induced male hepatic vitellogenin gene expression.

Induced male hepatic vitellogenin should not be indicative of reproductive dysfunction *per se*. There have been reports of either no detrimental effects of vitellogenin-induced males on reproductive success in fish (Mills *et al.* 2003; Filby *et al.* 2010) or even stimulated reproductive output in *P. promelas* exposed to low concentrations (1 ng L^{-1}) of EE_2 (Jobling *et al.* 2003). A prolonged exposure to xenoestrogens *in situ*, however, may lead to intersex and reproductive disorder and be detrimental to fish populations (Kidd *et al.* 2007).

Hepatic vitellogenin gene expression was induced in male fish, which might indicate that the chemical composition of the effluent became more estrogenic due to OBP formation. This consequence has also been illustrated in earlier studies of an ozonated hospital effluent (Maletz *et al.* 2013) and E2 (Bila *et al.* 2007). The vitellogenin induction was, however, an unexpected result in light of previous studies showing the opposite – reduced vitellogenic responses in post-ozonated STP effluent as compared to the untreated effluent (Gunnarsson *et al.* 2009b; Stalter *et al*. 2010; Cuklev *et al.* 2012).

Another explanation for the increased fecundity could be that the ozonated STP effluent represented an optimal breeding environment for the fish compared to the effluent and tap water. An early study on different sewage effluent disinfectant technologies and the effects of treated effluents in fathead minnow (*Pimephales promelas*) reported highly successful reproduction in the ozonated effluent, attributed to an optimal breeding environment for the fish (Ward & DeGraeve 1978).

4.1.3 Zebrafish embryo-larvae developmental and behavioral responses

No significant alterations of the analyzed zebrafish embryo-larvae endpoints (proportion dead and malformed embryos and larval locomotor activity) between the treatments were detected. Previous studies of an ozonated membrane-filtered sewage effluent have reported adverse effects in rainbow trout (*Oncorhynchus mykiss*) embryo-larvae, presumably by transformation product formation (Stalter *et al.* 2010). Newly fertilized Japanese medaka (*Oryzias latipes*) embryos exposed to sewage collected from a Chinese STP ozonated in a laboratory setting displayed affected hatching rate and incidence of death and malformations in ozone concentration-response manner (Cao *et al.* 2009). Likewise, Yan *et al.* (2014) observed increasing embryo mortality and larval deformity in *O. latipes* exposed to ozonated sewage, due to aldehydic compound build-up from ozonation. Sewage ozonation has, on the other hand, also alleviated adverse effects from exposure to the non-ozonated sewage in fish embryo-larvae (Lundström *et al.* 2010; Margot *et al.* 2013; Sun *et al.* 2017).

4.1.4 Adult zebrafish behavioral response

The behavioral assay was performed on the final day of the 21-day exposure. Directly after transfer from its home aquarium to a novel tank, each zebrafish was recorded by an above-mounted video camera for 10 minutes. Fish exposed to the ozonated STP effluent exhibited a significantly lower swimming activity during the first minute of the swimming activity test as compared with tap water control exposed individuals (ANOVA $p < 0.0001$). Thus, the less active fish exhibited a more pronounced 'freezing' behavior when introduced to a novel, unknown environment. No difference in swimming activity between the control and the non-ozonated effluent treatment group was detected. This result could indicate a stress-related behavioral phenotype in the fish exposed to the ozonated effluent. Reduced swimming activity in fish suddenly put into a novel environment (i.e. aquarium) may be interpreted as a stress response (Kalueff *et al.* 2013).

Stress is an important factor for survival in hostile settings, but chronic stress may produce subsequent adverse effects (Wendelaar Bonga 1997). Stressed fish exhibit induced levels of the hormone serotonin (Winberg *et al.* 1992). Serotonin modulates the hypothalamus-pituitary-interrenal axis in fish. Furthermore, it also stimulates the reproductive neuroendocrine axis in fish by increasing the release of gonadotropins from the pituitary gland, which controls gonad maturation and egg release (Prasad *et al.* 2015). Whether endocrine disruption of the serotonergic system was influencing the observed fecundity and stress effects in Paper I remains speculative. However, recent experimental evidence supports the notion that STP effluent ozonation may induce brain serotonin concentrations in exposed fish (Maya *et al.* 2018). Whether these effects would cause adverse impacts in wild fish populations is difficult to predict. It should also be reiterated that the reproductive and behavioral effects on adult zebrafish in Paper I was observed at 100% effluent concentration. In the environment, effluents are diluted in the recipient stream leading to lower concentrations of micropollutants.

4.2 Bench-scale ozonation of carbamazepine, diclofenac, and oxazepam (Paper II)

The second part of the thesis work (Paper II) aimed to test embryotoxicity outcomes of ozone treatment of individual pharmaceuticals in a controlled laboratory setting. The biological outcomes of the study presented in Paper I were resulting from exposure to a mixture of at least 24 ozonated APIs. Three of these, carbamazepine, diclofenac, and oxazepam were selected for testing in Paper II. These particular pharmaceuticals were chosen because they cover three

different classes of drugs (i.e. an anticonvulsant drug, a nonsteroidal anti-inflammatory drug (NSAID), and a benzodiazepine) with different modes of action and biological targets. Furthermore, they exemplified pharmaceuticals both degradable (carbamazepine and diclofenac) and not easily degradable by ozonation (oxazepam) in the STP effluent ozonation study (Paper I).

Paper II combined the embryotoxicity effect screenings with chemical screenings. The chemical screenings were performed to elucidate ozone removal efficiencies of the pharmaceuticals in the bench ozonation set-up, as well as investigate the formation of four putative carbamazepine OBPs. Ozonated tap water was also tested in each of the three pharmaceutical ozonation studies. No adverse biological responses were detected, indicating that residual ozone did not cause toxicity in the experimental setup.

4.2.1 Ozonation removal efficiencies

Ozonation for 1 min removed 20 ± 3.4% carbamazepine, 25 ± 16% diclofenac, and 2.0 ± 4.4% oxazepam. Increasing ozone dosage time improved removal of the parent compound in all three tested pharmaceuticals. Ozonation for 10 min removed 90 ± 11% carbamazepine, 97 ± 3.8% diclofenac, and 19 ± 5.7% oxazepam. These results are comparable to the ozone removal efficiencies reported in Paper I, suggesting that the experimental ozonation setup in Paper II served as a reasonable model of a large ozonation facility.

Chemical analysis revealed a discrepancy between nominal and measured concentrations of carbamazepine and oxazepam. The nominal concentrations corresponded with the measured concentrations on average by 59% for carbamazepine and 81% for oxazepam. These deviations were probably due to issues of solubility and precipitation in water for carbamazepine and Dimethyl sulfoxide (DMSO) for oxazepam. Measured concentrations of diclofenac were in good agreement with nominal concentrations.

4.2.2 Diclofenac

Diclofenac is a nonsteroidal anti-inflammatory drug used for the treatment of inflammation and related pain. Exposure at medium (7.4 mg L^{-1}) and high (15 mg L^{-1}) diclofenac concentrations caused pericardial- and yolk-sac edemas, as well as absent systemic circulation, at 48 hpf. The highest concentration (15 mg L^{-1}) exposure also caused a reduced heart rate by ~50%. Subsequently, this treatment group exhibited 100% mortalities at 144 hpf. Hatching time was not affected in any treatment group, however embryos in treated in the highest

concentration all died before hatching. No behavioral effects in any treatment group were detected for diclofenac in the locomotor activity assay.

Diclofenac toxicity was completely abolished after ozonation. The proportion affected individuals (81%) exposed to the lowest diclofenac concentration at 144 hpf, was reduced to the control group level after 1 min ozonation. Diclofenac ozonated for 10 min did not produce any toxicity in any endpoint. A Microtox (*Vibrio fischeri* NRRL B-11177 strain) *in vitro* study has previously indicated a reduction in diclofenac toxicity following ozonation, not unlike the outcome presented in Paper II (Coelho *et al.* 2009). There are, however, to our current knowledge no previously published studies on fish embryotoxicity of ozonated diclofenac. Diclofenac ozonation did not form toxic OBP, based on the results from Paper II.

4.2.3 Oxazepam

Oxazepam is an anxiolytic psychoactive drug used to treat anxiety disorders. No alterations in zebrafish lethal or sublethal endpoints were detected in any treatment group, either pre- or post ozonation. The larvae locomotor activity screening did not give rise to any deviations in behavior between the different concentrations. However, ozonation of the highest ozone concentration for 10 min (reducing the oxazepam concentration from 7 to 6.1 mg L^{-1}) caused hypoactivity during dark conditions.

Oxazepam is known to produce behavioral effects (increased boldness) in adult wild European perch at a concentration of 910 µg L^{-1} (Brodin *et al.* 2013). Embryonal life stages of fish however seem to be less sensitive to oxazepam exposure than adult fish. A reduced swimming activity during dark periods has been recorded previously in larval *O. latipes* exposed to 10 mg L^{-1} oxazepam (Chiffre *et al.* 2016). Oxazepam is a $GABA_A$ receptor activator, an anxiolytic benzodiazepine which gives rise to GABAergic behavioral modifications. Since larvae activity is induced by dark periods (an anxiety-like response) in the larvae swimming activity assay, the hypoactivity illustrated in Paper II and other studies may thusly be explained by an anxiolytic mode of action. One possibility of this response could be the formation of OBPs potentiating the anxiolytic properties of oxazepam in the exposed zebrafish embryos. The results of Paper II thusly indicated that ozonation of oxazepam may increase its anxiolytic properties, which may lead to effects on subsequent survivability.

4.2.4 Carbamazepine

Carbamazepine is a drug used for the treatment of epilepsy and related symptoms. Carbamazepine exposure resulted in embryotoxicity at 144 hpf in the medium (30 mg L^{-1}) and highest (50 mg L^{-1}) treatment groups with 88% and 100% affected individuals respectively, mostly due to occurrence of pericardial edema. Carbamazepine also significantly reduced the heart rate at 48 hpf in the highest treatment group (50 mg L^{-1}), and all individuals in this group were dead before hatching. The pre-hatching mortalities were preceded by the incidence of pericardial and yolk-sac edemas recorded at 48 hpf. The locomotor activity assay at 144 hpf did not show any significant effects on larvae behaviors in surviving unaffected larvae exposed to any treatment. Previous studies on zebrafish embryotoxicity of carbamazepine have reported results in line with Paper II. Beker van Woudenberg *et al.* (2014) reported that the most sensitive endpoint observed in zebrafish embryos exposed to carbamazepine was a delayed onset of hatching (72 hpf, EC_{50}: 45.5 mg L^{-1}) and pericardial edema (96 hpf, EC_{50}: 52 mg L^{-1}). Besides embryotoxicity in zebrafish, carbamazepine (100 µg L^{-1}) has also been shown to cause reproductive toxicity in adult Chinese rare minnows *Gobiocypris rarus* (Yan *et al.* 2018). Since carbamazepine is a highly persistent pharmaceutical in the aquatic environment, its removal from STP effluents should be considered a priority (Björlenius *et al.* 2018). However, as became evident in Paper II, ozonation may be an unsuitable option for carbamazepine abatement.

Ozonation of carbamazepine increased the toxicity in all endpoints. The lowest observed effect concentration (LOEC) of carbamazepine based on proportion affected (dead and malformed) embryos was reduced from the medium (30 mg L^{-1}) to the lowest (17 mg L^{-1}) concentration following ozonation for 10 min. The heart rate and hatching time followed the same pattern of increased toxicity after 10 min carbamazepine ozonation. Increased carbamazepine toxicity post-ozonation has been illustrated in earlier *in vitro* cell-based studies (Dwivedi *et al.* 2018; Han *et al.* 2018) and a macroinvertebrate study (Heye *et al.* 2016).

Since carbamazepine ozonation caused increasing embryotoxicity, four putative transformation products were included in the chemical analysis in Paper II. The rationale for measuring these specific compounds was that analytical standards were commercially available. Two of the OBPs, CBZ-EP and DI-CBZ, increased in concentration with increasing ozonation dosing. CBZ-EP was detected in all ozonated carbamazepine samples, formed increasingly in relation to increasing ozone dosage. CBZ-EP is the main therapeutically active metabolite of carbamazepine formed by hepatic cytochrome p450 (Breton *et al.* 2005). Based on the results in Paper II, it can also formed by ozonation. In a

previous study comparing the toxicity of carbamazepine and CBZ-EP in exposed macroinvertebrate (*Chironomus riparius*), CBZ-EP was found to be more than five times more toxic (Heye *et al.* 2016). Research has indicated how carbamazepine is biotransformed to CBZ-EP in fish, illustrating the concern of potential biological effects occurring in non-target species (Valdés *et al.* 2016). The second most detected carbamazepine OBP in Paper II was DI-CBZ. All carbamazepine treatment solutions, ozonated and not ozonated, contained concentrations of DI-CBZ exceeding LOQ. This may indicate that DI-CBZ was present as a contaminant in the carbamazepine analytical standard (manufacturer reported purity ≥ 98%). The quantified concentrations did however increase with increasing ozone dose, indicating that it was indeed formed by ozonation.

The highest concentration among the screened OBPs was for CBZ-EP, with 3.5 mg L^{-1} in the highest carbamazepine solution ozonated 10 minutes. DI-CBZ was the second most formed OBP, with a maximum concentration at 0.27 mg L^{-1}. CBZ-EP and DI-CBZ were tested for embryotoxicity in Paper III along with two other carbamazepine OBPs inferred from literature, 1-(2-benzaldehyde)-4-hydro-(1*H*,3*H*)-quinazoline-2-one (BQM), and 1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione (BQD).

4.3 Embryotoxicity and stability of carbamazepine OBPs (Paper III)

Since carbamazepine embryotoxicity was induced following ozonation in Paper II, the thesis work progressed by testing individual carbamazepine OBPs separately and in a mixture. Four OBPs were selected based on information from chemical screening in Paper II (CBZ-EP and DI-CBZ) and in the literature (BQM and BQD; McDowell *et al.* (2005)). Ozonation of 17.5 mg L^{-1} carbamazepine for 10 minutes (0.29 mg L^{-1} peak dissolved O_3 concentration) produced 0.5 mg L^{-1} CBZ-EP, 0.02 mg L^{-1} DI-CBZ, 2.2 mg L^{-1} BQM and 5.5 mg L^{-1} BQD. The CBZ-EP concentration corresponded well to the quantified concentration in Paper II (0.5 mg L^{-1}). DI-CBZ was however quantified at a lower concentration (0.02 mg L^{-1}) compared with results obtained in Paper II (0.2 mg L^{-1}). A higher ozone dosage, due to the use of a higher capacity oxygen injection apparatus, may explain this discrepancy (~0.15 mg L^{-1} in Paper II, ~0.3 mg L^{-1} in Paper III). Chemical analysis of the pre-ozonated carbamazepine solution detected 0.04 mg L^{-1} DI-CBZ. Presence of DI-CBZ in the pre-ozonated carbamazepine was, as previously mentioned, also observed in Paper II.

Three embryotoxicity endpoints (proportion of affected embryos (dead and malformed) at 144 hpf, heart rate, and time until hatching) were screened in the zebrafish embryo toxicity test assays. CBZ-EP (0.8–2.6 mg L^{-1}) and DI-CBZ

(0.1–0.4 mg L^{-1}) exposure did not cause embryotoxicity at the tested concentrations. The concentration-ranges were based on the chemical analysis in Paper II, where 0.5 mg L^{-1} CBZ-EP and 0.2 mg L^{-1} DI-CBZ were quantified in ozonated carbamazepine. Three nominal concentrations corresponding to half (0.5×), equal (1×), and twice (2×) that of the previously quantified concentrations were used. It was consequently deducted that CBZ-EP and DI-CBZ formation were not the reasons for induced embryotoxicity following carbamazepine ozonation.

The focus then shifted to BQM and BQD. Once again, three nominal concentrations based on the quantified concentrations following ozonation (0.5×, 1×, and 2×) were used as treatments for each compound. The 1× treatments (BQD: 1.9 mg L^{-1}, BQM: 1.8 mg L^{-1}) and 2× treatments (BQD: 6 mg L^{-1}, BQM: 3.5 mg L^{-1}) caused reduced heart-rate and prolonged hatching time, thus explaining these responses in embryos exposed to ozonated carbamazepine. The 2× concentrations induced ozonated carbamazepine-like malformations in the embryos (lack of swim bladder inflation and pericardial- and yolk sac edemas), while the 0.5x and 1x treatments did not.

A mixture was prepared and tested for embryotoxicity in order to examine the joint effect of the four quantified carbamazepine OBPs. The mixture was designed to contain concentrations of each OBP relative to its respective concentration formed in ozonated carbamazepine in Paper II (regarding CBZ-EP and DI-CBZ) and Paper III (regarding BQD and BQM). Three mixture concentrations were tested. The lowest concentration 0.5× (CBZ-EP: 0.7 mg L^{-1}, DI-CBZ: 0.1 mg L^{-1}, BQD: 1.5 mg L^{-1}, BQM: 0.8 mg L^{-1}) did not cause any embryotoxic responses in any of the three tested endpoints. The 1× concentration (CBZ-EP: 1.9 mg L^{-1}, DI-CBZ: 0.2 mg L^{-1}, BQD: 2.6 mg L^{-1}, BQM: 1.6 mg L^{-1}) caused significant induction of affected (dead and malformed) embryos. A majority of the affected embryos displayed similar malformation types as those exposed to ozonated carbamazepine in Paper II (i.e. pericardial edema and/or lack of swim-bladder inflation). The highest mixture concentration 2× (CBZ-EP: 4.3 mg L^{-1}, DI-CBZ: 0.5 mg L^{-1}, BQD: 7.7 mg L^{-1}, BQM: 3.7 mg L^{-1}) caused 100% embryo mortalities before 24 hpf. Heart-rate was not affected in the 0.5× treatment. Meanwhile, the 1× treatment group exhibited a reduced heart-rate, similar to embryos exposed to ozonated CBZ in Paper II. The hatching time was however not affected by any mixture treatment.

In order to test the stability of the carbamazepine OBPs, the solutions were stored under laboratory conditions (22 °C) for two weeks during which time the concentrations were measured. Furthermore, the embryotoxicity of ozonated carbamazepine was tested repeatedly over a two-week storage of the test solution (testing after 0 h, 1 h, 24 h, 1 week, and 2 weeks of storage). This was done to

determine the stability of the OBPs in the experimental water and if the embryotoxicity induced after ozonation of carbamazepine persists even after prolonged storage, and consequently indicate that the OBPs formed could be persistent in the environment. All analyzed OBPs remained at stable concentrations during the two-week storage period except BQD, which declined by 94%. Meanwhile, embryotoxicity remained in all tested storage time points, decreasing from ~95% directly after ozonation to ~75% after two week storage. Since BQM and BQD have previously been detected in an ozonated effluent (McDowell *et al.* 2005), the toxic and persistence properties of BQM should be viewed as potentially problematic and in need of further investigation.

4.4 General discussion

As presented within this thesis, ozonation of effluents and pharmaceuticals can lead to the formation of toxic OBPs with potentially adverse effects in fish. In order to utilize ozonation to improve pharmaceutical residue elimination without risking increased toxicity, the method must be designed and operated with caution. Toxic OBPs need to be controlled for when applying ozonation as an additional treatment step at STPs. The OBPs, and their (eco)toxic effects, can often be removed from post-ozonated effluent by application of post-treatment filters (von Gunten, 2018; Völker *et al.* 2019). Formation of BQM and BQD from carbamazepine, which was determined to be the driver of post-ozonation toxicity induction in Paper III, has also been shown to be prevented by applying zeolite catalysts in the ozonation process (Rosal *et al.* 2008; Saeid *et al.* 2020). Further, experimental data has shown that a filter system (e.g. GAC) alone can be as effective as ozonation for removal of micropollutants including pharmaceuticals, but may be more expensive for full-scale implementation (Baresel *et al.* 2017).

An important question regarding additional sewage effluent treatment technologies is indeed their monetary and environmental costs. The energy required for advanced treatment technologies could lead to increased carbon dioxide emissions, but ozonation seems to be favorable in terms of carbon footprint compared with carbon filter systems (Baresel *et al.* 2017). Ozonation as a sewage treatment technology has also been problematized in the context of human health, since the air pollution resulting from ozonation may potentially counteract the projected positive impacts on water quality (Papa *et al.* 2013). The beneficial aspects of ozonation should, however, continue to increase as technological developments will improve energy efficiency (Papa *et al.* 2013). It must also be mentioned that the issue of pharmaceutical pollution in the aquatic environment may not just be tackled by end-of-pipe approaches such as

ozonation. Upstream actions, meaning mitigation measures such as overhauling prescription practices or implement urine separating toilets, could also be part of the solution (Blair, 2016).

In the Knivsta STP effluent (Paper I), carbamazepine was detected at a concentration of 0.2 μg L^{-1}, which would lead to a theoretical BQM concentration of 0.08 μg L^{-1} and a BQD concentration of 0.02 μg L^{-1} post ozonation (based on calculated yields using the ozonation method in Paper III). Whether these concentrations would constitute a risk to the aquatic environment is not clear, however these concentrations are ~ 90 000 lower than the lowest observed effect concentrations in Paper III. This may demonstrate a limited risk to developing zebrafish embryos. However, aspects of bioaccumulative properties and long-term environmental effects, which are highly relevant since BQM seem to have persistent properties, is yet to be uncovered. Environmental monitoring and (eco)toxicity assessment of OBPs and other metabolic or biotransformation metabolites are challenging endeavors since these compounds rarely have commercially available analytical standards (Lee & Gunten, 2016). As such, *in silico* modeling of toxicophores (i.e. chemical structures with predicted toxic potency) arising post-ozonation has been emphasized as a useful method for 'flagging' potentially toxic OBPs (Escher & Fenner, 2011). Still, whole organism *in vivo* toxicity assessment will continue to be important to verify predicted toxic potencies of pharmaceutical OBPs.

The thesis work primarily used the zebrafish embryo model as a whole-organism *in vivo* model, which allowed rapid and data-rich testing of ozonated pharmaceuticals and isolated OBPs using a limited volume of test solution. The suitability and environmental relevance of the zebrafish embryo should however be objectively highlighted at this point. For instance, the metabolic activation of the tested chemicals may be lower in the embryo compared with the adult fish, thus leading to weaker sensitivity in zebrafish embryo tests (Klüver *et al.* 2011; Knöbel *et al.* 2012). Other factors such as the physiochemical properties of the tested compound may affect uptake in the embryonal life-stage, leading e.g. to lower internal concentrations in the embryos as compared with adult fish (Klüver *et al.* 2015). Still, the fish embryo toxicity *in vivo* model has been shown to accurately predict toxicity in adult fish for a multitude of micropollutants with different modes of action, indicating that it is a relevant model for (eco)toxicity hazard assessments (Belanger *et al.* 2013) In conclusion, it might not be prudent to directly extrapolate the results of the thesis work (Paper II and III) to the ecosystem level, as the embryotoxicity tests indicated adverse effects of the tested pharmaceuticals and OBPs at 1000−100 000 times the concentrations detected in the aquatic environment. Despite their relative lack of sensitivity, however, the embryotoxicity tests provided valuable information on the

diverging toxic outcomes following the ozonation of pharmaceuticals due to OBP formation.

5 Conclusions

This thesis has investigated the chemical and biological outcomes of ozonation in the context of pharmaceuticals in STP effluents. While chemical analysis revealed enhanced removal efficiencies of several pharmaceuticals, biological responses in zebrafish also became induced, illuminating the complexity of ozonation as an advanced sewage treatment technology. In Paper I, ozonation of the STP effluent removed 77% of the measured pharmaceuticals. The ozone-treated effluent did however give rise to unexpected potentially adverse effects such as induced vitellogenin gene expression in male fish, and an anxiety-like stress response in these fish was observed. These biological responses may possibly indicate effects due to OBP formation. Further, fecundity was 2-fold higher in fish exposed to the ozonated effluent compared to the non-ozonated effluent and the tap water control group.

Whether the increased fecundity constituted an adverse or beneficial outcome remains speculative. Diverging zebrafish embryotoxicity outcomes were then observed following ozonation of carbamazepine, diclofenac, and oxazepam in a bench-scale laboratory ozonation reactor (Paper II). While diclofenac embryotoxicity was eliminated, carbamazepine and oxazepam showed induced embryotoxic effects following ozonation. Carbamazepine OBPs were then isolated and tested for embryotoxicity individually and in mixture (Paper III). The study revealed the drivers of carbamazepine post-ozonation embryotoxicity as being BQM and BQD, thus directly associating OBPs to adverse biological effects of pharmaceutical ozonation. Besides, BQM was shown to be persistent over a two-week stability test under laboratory conditions, indicating that carbamazepine OBPs could be environmentally relevant toxic compounds needing attention. Efficient formation prevention and removal of OBPs from ozonated sewage effluents should ultimately be ensured in order to avert potentially increasing environmental risks.

6 Future perspectives

There are considerable knowledge gaps regarding the environmental effects and fate of pharmaceutical OBPs and other transformation products originating from water treatment. Future work on this issue requires an integrated multidisciplinary approach combining methods from analytical chemistry and ecotoxicology, as has been utilized in this thesis.

- Future efforts should proceed to gather mechanistic data of OBPs, in order to explain how ozone-mediated oxidation of micropollutants increase toxicity.
- Bioconcentration factors and bioaccumulative properties of potentially problematic OBPs (e.g. BQM) should be investigated
- Long term exposure and multigenerational testing at environmentally relevant concentrations could add valuable information on the potential environmental risks of OBPs.

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Popular science summary

Most people are aware that old, unused, medicine should be returned to the pharmacy, not sent down the toilet. Still, about half of all medicine we consume goes right into the aquatic environment through the sewage. It happens since the body excretes pharmaceutical residues in urine and feces after treatment. The sewage treatment plants cannot efficiently remove pharmaceutical residues from sewage water. Therefore we may count on that residues of all pharmaceuticals human and animals consumes ultimately reaches the aquatic environment. Pharmaceuticals can affect aquatic organisms since they share many if not most of the biological receptors that the pharmaceuticals interact with. For example, anti-anxiety medication can make perch less intimidated by their predators, and contraceptive pills can cause male fish to reverse sex and produce eggs in their testis.

Improvement of sewage treatment has been urged, since it has been shown that pharmaceuticals can affect fish and other aquatic organisms. Ozonation of sewage water could be a method to prevent pharmaceutical pollution in the environment. Ozone is a powerful gaseous oxidant which attacks the pharmaceutical molecule and degrades it. In 2014, a full-scale ozonation step was constructed at the Knivsta municipal sewage treatment plant in Sweden in order to study the removal efficiency of pharmaceuticals. Out of 105 pharmaceuticals screened for, 24 could be found in the sewage effluent before ozonation. Ozonation reduced the concentrations of these by 77%. Meanwhile, the biological effects of the sewage water before and after ozonation was studied in fish (zebrafish, *Danio rerio*). The fish were divided into three experimental groups during the three-week study. It was shown that fish in the ozonated sewage water spawned twice as many eggs as in the two other groups. Whether this outcome meant that the fish fared better or worse than the other groups us difficult to determine. The male fish in the ozonated sewage group also displayed an upregulated expression of an estrogen-sensitive gene which indicated that ozonation may have created more estrogenic-like chemicals in the sewage water.

The behavior of the fish in the ozonated sewage effluent was also affected. Fish tended to be more stressed by being separated from their home aquaria at the end of the study. These results may point to that ozonation can change the chemical composition of sewage water, and thereby affect fish in new ways.

Ozone hardly ever breaks down pharmaceuticals completely, and it has been shown that ozonation can form so-called byproducts. How toxic these compounds are is largely unknown. That is why the three common pharmaceuticals diclofenac, carbamazepine, and oxazepam were studied regarding how ozone would affect their toxicity in zebrafish embryos. It was shown that diclofenac and carbamazepine were removed by more than 90% from water solution after ozonation, while oxazepam was less easily broken down (20%). Diclofenac ozonation also effectively removed its toxicity. On the flip side, the toxicity of carbamazepine and oxazepam increased, which was surprising and a bit concerning. Carbamazepine byproducts have identified earlier, and with the help of this information we could isolate and test four different carbamazepine byproducts. This enabled an additional study on zebrafish embryos where we for the first time could prove that two compounds, BQM and BQD, were responsible for the increased toxicity of carbamazepine following ozonation.

Ozonation of pharmaceuticals can create toxic byproducts, which is problematic. We should avoid creating more damage than good when we improve the treatment of sewage water. Fortunately, it seems that byproducts can be broken down by microorganisms in filters that the sewage water can pass after ozone treatment. Some add-ons to the ozonation process, such as catalysts, can prevent the creation of byproducts. The problem of pharmaceutical residues in the environment will however probably need more types of solutions. For instance, the development of new drugs that more easily breaks down into non-toxic byproducts in sewage, or collection and separate treatment of urine in toilets. The research aiming for a cleaner aquatic environment, free from pharmaceuticals, continues.

Populärvetenskaplig sammanfattning

De flesta känner till att gamla oanvända mediciner ska skickas tillbaka till apoteket, inte ner i toaletten. Ändå går i princip hälften av alla läkemedel vi äter rakt ut i vattenmiljön via avloppet. Det sker eftersom kroppen utsöndrar resterna av läkemedlen genom urin och avföring efter behandling. Avloppsreningsverken kan i dagsläget inte effektivt rena bort läkemedelsrester från avloppsvattnet. Därför kan vi räkna med att spår av all medicin som både människor och djur äter till sist når vattenmiljön. Läkemedel kan påverka vattenlevande organismer som i stort delar de biologiska receptorer som läkemedlen verkar på. Till exempel kan ångestdämpande mediciner göra abborrar mindre rädda för de rovdjur som jagar dem, och östrogena p-piller kan orsaka att hanliga fiskar byter kön och börjar bilda ägg i testiklarna.

Då man har sett att läkemedel kan påverka fiskar och andra vattenlevande djur vill man förbättra reningen. Ozonering av avloppsvatten skulle kunna bli ett sätt att förebygga läkemedel i miljön. Ozon, en kraftfull oxidant i gasform, angriper läkemedelsmolekylen och bryter ned den. Vid Knivsta reningsverk byggdes 2014 en ozoneringsanläggning i full skala för att testa hur mycket av läkemedlen i avloppsvattnet som kunde brytas ned. Av 105 uppmätta läkemedel kunde 24 upptäckas i avloppet. Ozoneringen reducerade koncentrationerna av dessa med 77 %. Under tiden undersöktes effekterna av avloppsvattnet före och efter ozonering på fisk (sebrafisk, *Danio rerio*). Fiskarna delades in i tre försöksgrupper under försöket som varade i tre veckor. En i vanligt avloppsvatten, en i ozonerat avloppsvatten och en kontrollgrupp i vanligt kranvatten. Det visade sig att fiskarna i det ozonerade avloppsvattnet lekte bättre, och producerade dubbelt så många ägg än fiskarna i de två andra grupperna. Om det berodde på att fiskarna i det ozonerade avloppsvattnet mådde bättre än de andra grupperna eller sämre är dock svårt att avgöra. De hanliga fiskarna från gruppen i det ozonerade avloppsvattnet uppvisade även ett ökat genuttryck av en östrogen-känslig gen vilket indikerade att ozoneringen kan ha bildat fler östrogen-lika ämnen i avloppsvattnet. Vidare så var beteendet i fiskarna i det

ozonerade avloppet påverkat, då de verkade mer stressade av att placeras ensamma i ett nytt akvarium i slutet av försöket. Dessa resultat kan peka på att ozoneringen kan förändra den kemiska sammansättningen i avloppsvattnet, och därigenom påverka fiskar på nya sätt.

Ozonets nedbrytning av läkemedel är sällan fullständig, och det har visat sig att ozonering kan bilda restprodukter. Hur giftiga (toxiska) dessa ämnen är finns det dock relativt lite kunskap kring. Därför studerades de tre vanliga läkemedlena diklofenak, karbamazepin och oxazepam och hur ozonering av dem påverkade deras toxicitet i sebrafisk-embryon. Det visade sig att diklofenak och karbamazepin kunde tas bort från vattnet med över 90%, medan oxazepam var svårare att bryta ned (ungefär 20%). Ozoneringen av diklofenak tog även effektivt bort dess giftiga effekter på embryona. Däremot orsakade ozonering att de skadliga effekterna av karbamazepin och oxazepam ökade, vilket var överraskande och lite oroande. Karbamazepinets restprodukter har tidigare identifierats, och med hjälp av denna information kunde vi isolera och testa fyra olika restprodukter av karbamazepin. Detta möjliggjorde en till studie på sebrafiskembryon där vi för första gången kunde bevisa att två ämnen, BQM och BQD, stod för de ökade skadliga effekterna efter ozonering av karbamazepin.

Att ozonering av läkemedel bildar toxiska restprodukter är problematiskt. Vi bör undvika att skapa mer skada än nytta när vi förbättrar reningen av avloppsvatten. Lyckligtvis verkar det som att restprodukter kan brytas ned med hjälp av mikroorganismer i filter som avloppsvattnet passerar efter ozoneringen. Även olika tillägg i ozoneringen, som till exempel katalysatorer, kan förhindra uppkomsten av restprodukter. Problemet med läkemedelsrester i miljön kommer dock förmodligen behöva flera olika typer av lösningar. Exempelvis att utveckla nya mediciner som lättare bryts ned till icke toxiska restprodukter, och uppsamling av urin i toaletter som kan renas separat. Forskningen som syftar till en renare vattenmiljö, fri från läkemedelsrester, går framåt.

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I

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Effects of ozonated sewage effluent on reproduction and behavioral endpoints in zebrafish (*Danio rerio*)

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ABSTRACT

Pharmaceutical residues and other micro-contaminants may enter aquatic environments through effluent from sewage treatment plants (STPs) and could cause adverse effects in wild fish. One strategy to alleviate this situation is to improve wastewater treatment by ozonation. To test the effectiveness of full-scale wastewater effluent ozonation at a Swedish municipal STP, the added removal efficiency was measured for 105 pharmaceuticals. In addition, gene expression, reproductive and behavioral endpoints were analyzed in zebrafish (*Danio rerio*) exposed on-site over 21 days to ozonated or non-ozonated effluents as well as to tap water. Ozone treatment (7 g O_3/m^3) removed pharmaceuticals by an average efficiency of 77% in addition to the conventional treatment, leaving 11 screened pharmaceuticals above detection limits. Differences in biological responses of the exposure treatments were recorded in gene expression, reproduction and behavior. Hepatic vitellogenin gene expression was higher in male zebrafish exposed to the ozonated effluent compared to the non-ozonated effluent and tap water treatments. The reproductive success was higher in fish exposed to ozonated effluent compared to non-ozonated effluent and to tap water. The behavioral measurements showed that fish exposed to the ozonated STP effluent were less active in swimming the first minute after placed in a novel vessel. Ozonation is a capable method for removing pharmaceuticals in effluents. However, its implementation should be thoroughly evaluated for any potential biological impact. Future research is needed for uncovering the factors which produced the in vivo responses in fish.

1. Introduction

Pharmaceutical residues in domestic and industrial wastewaters pollute the aquatic environment worldwide (aus der Beek et al., 2016; Fent et al., 2006; Hughes et al., 2013). Conventional sewage treatment plants (STPs) are not designed to efficiently remove pharmaceuticals (Verlicchi et al., 2012). Some pharmaceuticals, such as oxazepam, bioaccumulate in several trophic levels of aquatic food webs (Lagesson et al., 2016). This suggest that pharmaceutical contamination of aquatic ecosystems could affect biodiversity and potentially also ecosystem functioning of recipients (Jonsson et al., 2015). In line with this, several studies have shown that environmentally relevant pharmaceutical concentrations can affect ecologically important traits in fish. For example, fish exposed to synthetic progestins at low ng L^{-1} concentrations may downright fail to reproduce (Zeilinger et al., 2009). Estrogens, natural and synthetic, induce female specific hormone expression (vitellogenin, VTG) in males and produce intersex individuals with diminished reproductive function at levels found downstream from some sewage treatment plants (Kidd et al., 2007; Örn et al., 2016). The ubiquitous non-steroid anti-inflammatory drug diclofenac cause kidney damage in fish exposed at near environmentally relevant concentrations (Näslund et al., 2017). Psychoactive pharmaceuticals are also present in water bodies influenced by STP effluents (Grabicova et al., 2017). Lab and field studies have shown that oxzepam, an anxiolytic benzodiazepine, impacts fish behavior and could in turn affect whole populations (Brodin et al., 2013; Klaminder et al., 2016).

Ozonation is emerging as a potential method to improve wastewater treatment (Eggen et al., 2014). Ozone and ozone-generated OH radicals react readily with molecules of electron rich moieties, and have the capacity to decrease concentrations of pharmaceuticals and other

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micro-contaminants, often with high efficacy (Lee and von Gunten, 2016). Diclofenac and ethinylestradiol, for example, have been shown to be efficiently removed (~99%) by ozone (Hollender et al., 2009; Snyder et al., 2006; Sun et al., 2017; Ternes et al., 2003). It is however important to highlight that ozonation of the parent compound does not necessarily cause total mineralization of the compound. Partially oxidized ozonation by-products may have deleterious effects on aquatic biota such as fish (Prasse et al., 2015). Bromate and *N*-Nitrosodimethylamine (NDMA) are known ozonation by-products of toxicological concern (Zimmermann et al., 2011). Estrogenic chemicals are also partially degraded and generates ozonation by-products which could induce potentially adverse effects (Pereira et al., 2011).

Since the nature of STP effluents and ozonation strategies fluctuates strongly depending on season, abiotic conditions, time and location, it is imperative to gather as much knowledge of these different settings as possible. Previous studies have reported various outcomes from ozonated STP effluent exposure on fish. Examples from studies on rainbow trout larvae has suggested that ozone may increase (Stalter et al., 2010) or decrease (Margot et al., 2013) STP effluent toxicity. Samuelsson et al. (2011) showed consistent changes in the blood plasma composition of fish exposed to sewage effluents treated with a high (15 g O_3/m^3) but not a low (5 g O_3/m^3) concentration of ozone. To what extent it represented an adverse effect is not known. While the capability of ozone to remove pharmaceuticals and other micro-contaminants from effluents is promising, the biological effect outcome is of great importance to study in order to safeguard the quality of the aquatic environment. The present study investigated *in vivo* responses in zebrafish (*Danio rerio*) exposed on site at a lab located in Knivsta STP, Sweden, equipped with a parallel full scale ozonation stage. Chemical analysis was performed in order to assess the efficiency of ozone to reduce pharmaceutical concentrations in the conventionally treated STP effluent. The major aim of the work was to detect endocrine, reproductive and behavioral effects in fish and its progeny exposed to the conventional STP effluent. Furthermore, we sought to elucidate if any such effects were either abated or amplified by effluent ozonation.

2. Materials and methods

2.1. Knivsta STP

Knivsta STP is located 50 km north of Stockholm, Sweden. The wastewater is collected in a combined sewer system. Knivsta STP has a design capacity of 13 000 population equivalents (PE) (calculation base 70 g BOD_7 per PE and day) and in 2015 the plant treated municipal and to a smaller extent industrial wastewater from 12 000 PE in the Knivsta municipality. The treatment at Knivsta STP includes five major steps: pretreatment, primary sedimentation including chemical precipitation, biological treatment, secondary sedimentation and post-chemical precipitation. The pretreatment lines consist of screens with 3 mm bar distance followed by aerated grit chambers where ferric chloride solution is added for chemical precipitation of phosphor. Following pretreatment, primary sedimentation basins remove primary and chemical sludge. In the first of the two biological treatment steps, organic substances are degraded by the contact stabilization activated sludge concept. The activated sludge is separated in secondary sedimentation tanks. The wastewater is lifted to a second aerated biological treatment containing carriers to facilitate nitrification. Finally, the wastewater is distributed to flocculators where ferric chloride solution is added for removal of remaining phosphor. Subsequent sedimentation tanks remove the chemical flocs. In 2015, mean hydraulic retention time for wastewater in the treatment plant was 38 h, and the results for effluent standards were < 3 mg/L BOD_7, 9 mg/L total nitrogen, 0.11 mg/L total phosphor and 0.09 mg/L ammonia nitrogen (Roslagsvatten, 2016).

2.2. Ozonation plant

A full-scale ozonation step was designed, partly constructed and operated by the KTH Royal Institute of Technology to allow studies of ozonation and its effects on chemical and biological status of the effluent from Knivsta STP. The ozonation step was divided into two parallel lines which had a total maximum capacity of 560 m^3 effluent wastewater/h and included lifting pumps, production and injection of ozone, static mixers, contact tanks with compartments and final contact filters. Each line contained two lifting centrifugal pumps (APEX ISF C, Bristol, UK), one static mixer (NR Mixer, Statiflo International Ltd, UK), one 50 m^3 stainless steel contact tank with 5 m water depth and two compartments, one ozone destructors for off-gas (Primozone, Sweden), two contact filters with a total area of 2 × 12.7 m^2 filled with 1 m light expanded clay aggregates (Leca, Saint-Gobain Linköping, Sweden) for potential stripping or quenching of ozone residues. The ozone was produced from evaporated liquid oxygen with > 99.5% O_2 (YaraPraxair, Sweden) by an ozone generator with a maximum production capacity of 2.4 kgO_3/h (GM48, Primozone, Sweden). The addition of ozone was flow proportional up to 340 m^3/h at a dose of 7 gO_3/m^3, when the fixed maximum amount of 2.4 kg ozone/h was added giving a decreasing dose with further increasing flow. The water depth in the contact tanks was adjustable to 3, 4 or 5 m. After the ozonation step, the treated effluent passed the existing polishing pond before discharging into the receiving water, the small river Knivstaån.

2.3. Chemical analysis

A total 105 pharmaceuticals of various therapeutic classes were searched for in the effluent samples by online solid phase extraction and liquid chromatography tandem mass spectrometry (see Supplementary Material for full list). The method is described in detail in a previous study (Lindberg et al., 2014). In short, online extraction of filtered and acidified effluent samples was performed using an OASIS HLB (20 mm × 2.1 mm i.d., 15 µm particle size, Waters, Milford, Massachusetts, USA); chromatography was made by the use of a Hypersil GOLD aQ C18 (50 mm × 2.1 mm i.d., 5 µm particle size, Thermo Fisher Scientific, San Jose, CA, USA), or by a Varian Pursuit C18 (150 mm × 2.0 mm i.d., 5 µm particle size, Agilent Technologies, USA); pharmaceuticals were analyzed using a TSQ Quantum Ultra EMR triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) and quantified by means of internal standards.

2.4. Zebrafish exposure system

The mobile laboratory (isolated 10 ft ISO-container) was positioned adjacent to the conventional STP effluent pipeline and post-ozonation outgoing pipeline. Water from these two points were continuously pumped into two separate 60 L polyethylene plastic barrels inside the container. A third barrel was connected to the municipal drinking water network filtered by a carbon filter. An ozonated tap-water treatment group was not included in this study due to space constraints and technical limitations. Each barrel continuously supplied three aquariums at a continuous flow of 0.1 L/min. The experimental design thus consisted of three treatment groups (STP effluent, ozonated STP effluent and tap water control) with three replicates each. Fluorescent lights installed above the aquariums kept a 12:12 h light cycle. Each aquarium was equipped with an immersion heater maintained a 25 °C and the water was aerated by oxygen stones. The air temperature inside the laboratory container was kept at approximately 20 °C. All water and air temperatures were constantly monitored and logged using LoggerSoft software and probes (WebIQ AB, Sweden). Water quality parameters (pH, conductivity and ammonia) were regularly measured in all replicate aquariums.

The 21 day exposure study at the ozone facility in Knivsta took place between November 4th and November 25th 2015. Adult zebrafish (one

year old) were acclimatized and introduced into the 9 replicate aquariums in groups of 10 individuals (total n = 90). Each replicate consisted of 5 putative males and 5 putative females. The fish were fed once daily with Sera Vipan® (Sera, Germany) and observed for signs of stress, injury and/or mortality during the exposure period. The study was conducted in accordance with the ethical permit DNR79-2014 issued by the Animal Ethics Committee in Gothenburg, Sweden.

2.5. Fecundity and embryo toxicity tests

Fecundity measurements were performed during the last seven days of the exposure, in accordance to previous studies measuring fecundity in zebrafish (e.g. Lister et al., 2009). Metal mesh cages (5 mm mesh size) was placed in each aquarium to gather the fish and prevent them from consuming any eggs. All spawned eggs were siphoned from the bottom of the aquarium 30 min after the light was turned on (9.30 am). The number of fertilized, non-fertilized and damaged (inflated or lost chorion) eggs were counted in Petri dishes. Fertilized eggs (n = 24) from the first successful spawning event of each replicate (n = 3 per treatment) were transferred individually with 1 mL of water from its corresponding aquarium into 48 well plates. The plates were moved to our laboratory facilities (12:12 h light cycle, 26 °C air temperature). Mortality and malformations among the collected embryos were assessed at one, two and six days post fertilization (dpf).

2.6. Larvae locomotion test

The larvae locomotion (movement) response to alternating dark and light periods was tracked and recorded using the ZebraBox (ViewPoint, France) tracking system and ZebraLab software. The same 48 well plates containing six dpf larvae collected from the first successful spawning event (see Section 2.5) were used for the larvae locomotion test. Each plate was inserted into the ZebraBox chamber at between 12 pm and 6 pm. Each plate was acclimatized in the chamber for 10 min prior to recording to decrease handling-induced stress in the larvae. The lighting protocol involved four alternating 0% light intensity (henceforth denoted D1 and D2) and 100% light intensity (henceforth denoted L1 and L2) 10 min periods (D1 → L1 → D2 → L2). Tracking data consisted of individual total locomotor activity aggregated each minute for 40 min. Dead and/or malformed larvae were omitted from the tracking data analysis.

2.7. Adult behavioral test

At the termination of the 21 day exposure period all adult fish were individually transferred to non-transparent glass beakers (∅ 120 mm) along with 0.5 L of water from its corresponding aquarium. Four beakers placed on an IR light table were recorded simultaneously by an overhead mounted IR camera. Fish were distributed randomly from the treatment groups into the beakers. Total locomotor activity tracking data was acquired each minute by the ZebraLab software during a 10 min period after placement in the beakers.

2.8. Liver tissue sampling and gonad histology

After behavioral recording the fish were euthanized by a blow to the head and decapitated. The livers were dissected and snap-frozen in liquid nitrogen and kept at −70 °C for subsequent mRNA extraction. The trunk part of each individual fish were placed in separate plastic histology cassettes and fixed in phosphate-buffered formaldehyde. The samples were embedded in paraffin blocks after fixation and dehydration. Sections were cut and stained by haematoxylin–eosin and then evaluated under light microscopy. Males were classified as sexually mature if the testis contained mature spermatozoa. Females were classified as sexually mature if the ovary contained oocytes with vitellogenic yolk granules. Oocyte maturation ovary was further evaluated histologically by counting: 1) primary oocytes, 2) cortical alveolar oocytes and 3) vitellogenic oocytes (Selman et al., 1993). The proportions of these three stages of oocytes were determined in one representative longitudinal section from each female. The histological evaluations of gonads were done blindly without knowledge of treatments.

2.9. mRNA extraction and qRT-PCR

Liver tissues were homogenized by Precellys Evolution (Bertin instruments, France) system equipped with liquid nitrogen cooling. Each sample was placed in a 2 mL Precellys homogenizing tube with 500 µL RLT Plus buffer from the QiaSymphony RNA kit (Qiagen, Germany). After completion of the protocol (2 × 30 s at 6000 rpm, 30 s pause) 400 µL supernatant was transferred to new 2 mL Eppendorf tubes and inserted into the QiaSymphony SP instrument (Qiagen, Germany) for RNA extraction. The RNA integrity number (RIN) and RNA concentration of the samples were measured in a 2100 Bioanalyzer system (Agilent Technologies, USA). All samples had a RIN of between 7.4 and 9.5 (mean ± sd: 8.5 ± 0.4) and were included in the downstream qRT-PCR reactions.

Primers for the transcripts *ACTB* (Beta actin), *CYP1A1*, *ERα*, *VTG-2* (see Supplementary Material for information of oligonucleotide sequences, accession numbers and references) were ordered from and synthesized by Eurofins Genomics. Forward and reverse primers were added to each reaction respectively (200 nM each). Primer efficiency was confirmed before qRT-PCR of experimental samples began. Samples (100 ng template RNA per reaction) were run in duplicates in single reaction tubes with a total reaction volume of 20 µL (KAPA SYBR® FAST one-step qPCR kit) in a Rotor-Gene 3000 instrument (Corbett Research, Australia). A non-template control (NTC) was included in each batch. The qPCR protocol consisted of holding 42 °C for 5 min (reverse transcription), 95 °C for 3 min (enzyme activation) and 40 cycles of alternating 95 °C (3 s), 60 °C (20 s) and 72 °C (20 s). A melt curve analysis was performed following each reaction to ensure specific product amplification and check for primer dimer formation. Reaction products of the genes of interest were sent to Macrogen for sequencing. The resulting sequencing data was run on the NCBI Basic Local Alignment Search Tool (BLAST) to ensure product specificity. Relative mRNA expression was calculated by the E-ΔΔct method as described by (Livak and Schmittgen, 2001). Beta-actin (*ACTB*) was selected as a reference gene for normalization. Hepatic gene expression data from each male was used to calculate treatment means based on the three aquarium replicates.

2.10. Statistics

Data management, statistical modelling and figures was done using R software version 3.3.2 with the RStudio version 1.0.153 interface (R Core team, 2016; RStudio Team, 2016). Data were analyzed by one-way ANOVA and Tukey honest significance (HSD) test post-hoc test. Each aquarium (n = 3 per treatment) was considered an experimental unit. ANOVA and post hoc tests were considered significant if $p < .05$. Proportional data (e.g. oocyte stages distributions and larvae mortality) were arcsine-transformed prior to statistical modelling. Outliers were excluded from the mRNA expression data based on Grubbs' test (Grubbs, 1969; Komsta, 2011). Non-normal data were log-transformed in order to meet normality assumptions. Behavioral and cumulative egg production data were fitted as hierarchal mixed linear models using the nlme R package (Pinhiero et al., 2017). Group differences were assessed by least-square means comparison with Tukey's HSD p-value adjustment using the lsmeans R package (Lenth, 2016). To account for repeated measurements from the same subject an autoregressive correlation structure of lag 1 on the error term was set. All figures were made using the ggplot2 R package (Wickham, 2009).

Table 1
Concentrations of quantified pharmaceuticals in conventional STP effluent and after additional treatment by ozonation in Knivsta STP. The removal efficiency (%) was calculated based on the sum of API concentrations in each sample (n = 3). If the concentration of a pharmaceutical was below its limit of quantification (LOQ), it was given the value LOQ/2.

API	STP effluent (ng L^{-1})	Ozonation effluent (ng L^{-1})	Ozonation Removal efficiency (%)	LOQ (ng L^{-1})
Atenolol	633	209	56	15
Bisoprolol	126	48	49	4
Carbamazepine	207	< LOQ	97	7.5
Citalopram	351	< 25	90	20
Clindamycine	74	< LOQ	98	3
Codeine	219	< LOQ	94	20
Diclofenac	620	< LOQ	99	15
Eprosartan	214	< LOQ	96	15
Erythromycine	153	< LOQ	67	100
Fexofenadine	148	< LOQ	97	10
Flecanide	137	55	76	2
Fluconazole	200	147	24	7.5
Irbesartan	162	84	44	3
Memantine	12	4.8	52	4
Metoprolol	1244	406	63	15
Mirtazapine	68	< LOQ	84	20
Oxazepam	243	117	42	10
Ranitidine	125	< 19	84	20
Rosuvastatin	121	< LOQ	90	20
Sotalol	323	< LOQ	94	20
Sulfamethoxazol	258	< LOQ	97	15
Tramadol	298	60	87	20
Trimethoprim	173	< LOQ	99	4
Venlafaxine	178	< LOQ	67	20
Sum conc (ng L^{-1})	6392	1421	–	–
Average removal efficiency (%)	–	–	77	–
Number of APIs	24	11	–	–

3. Results and discussion

3.1. Ozone removal efficiency

Results from the chemical analysis of pharmaceuticals for the effluent and the ozonated effluent from Knivsta STP are summarized in Table 1. Of the 105 screened pharmaceuticals (complete list available as Supplementary Material), 24 were detected in the effluent samples from Knivsta STP during the exposure weeks. The ozone removal efficiency was on average 77% at an ozone dose of 7 g O_3/m^3. Of the 24 detected pharmaceuticals, 13 were removed to concentrations below the limit of quantification (LOQ). Total organic carbon (TOC) was on average 13.2 mg C/L in the effluent and 12.0 mg C/L in the ozonated corresponding to 8.6% removal of TOC. The presence and identities of ozonation by-products were not analyzed.

The average ozone removal efficiency of pharmaceuticals (77%) was slightly lower than the target value of 80%, probably caused by an insufficient ozone dose in relation to the concentration of organic substances in the effluent. The ozone dose was set to 7 g O_3/m^3 during the start-up of the ozonation plant which the corresponded to a specific ozone dose of 0.65 g O_3/g TOC in the feed to the ozonation plant resulting in higher than 80% pharmaceutical removal. During the exposure weeks in November the average TOC concentration in the effluent had increased from 11 to 13.2 mg C/L corresponding to 0.53 g O_3/g TOC in the feed to the ozonation plant but unfortunately the TOC was not analyzed at the time of the exposure, so the ozone dose was not adjusted.

3.2. Effects on male zebrafish hepatic gene expression

The male hepatic gene expression of the three genes *ERα*, *CYP1A1* and *VTG-2* is displayed in Fig. 1. *VTG-2* was induced 17-fold (p = .0196) in the ozonated STP effluent treatment group compared to the tap water control group. *CYP1A1* was induced two-fold compared to the tap water controls, but the difference was not statistically significant (p = .0825). Gene expression of *ERα* also showed a non-significant (p = .1077) tendency of induction in both the conventional and ozonated STP effluent.

The main driver of fish oocyte growth and maturation is the hypothalamic-pituitary-gonadal (HPG) axis. The gonadotropins, follicle stimulating hormone (FSH) and lutenizing hormone (LH) are key hormones in the HPG axis, present in plasma prior to and during spawning (Nagahama, 2002). The gonadotropins stimulate the ovary which initiates 17β-estradiol production and in turn signals the liver to produce vitellogenin (Clelland and Peng, 2009). Vitellogenin, or egg yolk precursor protein, is highly conserved in oviparous vertebrates and is produced in the female (Byrne et al., 1989). The serum estrogen concentration correlates with vitellogenin mRNA levels in fish hepatocytes

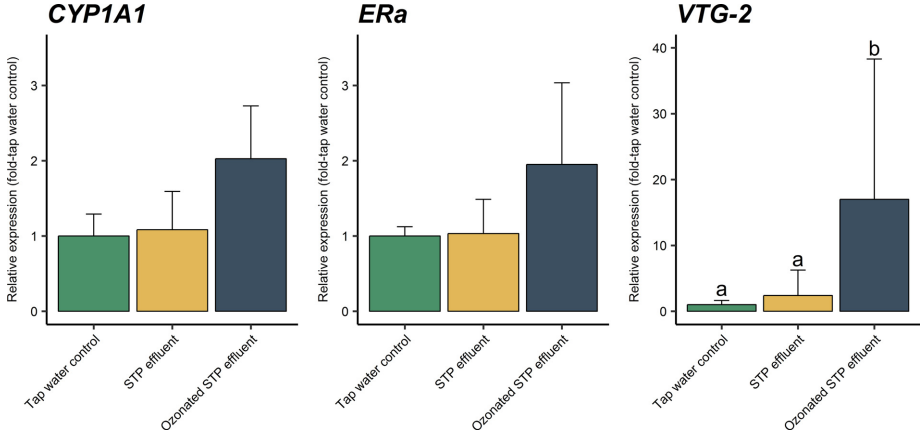

Fig. 1. Gene expression (fold change – tap water control) of *ERα*, *CYP1A1* and *VTG-2* in male zebrafish liver tissue (n = 3, mean + sd). Different letters indicate significant differences (Tukey's HSD p < 0.05) in the expression of *VTG-2* (ANOVA p = .0196). The gene expression data was normalized against the household gene *ACBT* (the expression of which was not altered by any treatment).

(Okumura et al., 2002). Estrogens bind to the estrogen receptors in hepatocytes and initiates vitellogenin gene expression (Hara et al., 2016). Induction of vitellogenin in male fish has been attributed to exposure of estrogenic xenobiotics, making it a sensitive biomarker for estrogenic exposure (Arukwe and Goksøyr, 2003). STP effluents typically contain a multitude of different xenobiotics with varying estrogenic potency. Such mixtures may have an additive effect on vitellogenin induction (Sumpter and Jobling, 1995). In the present study we did not detect the estrogenic parent compounds estradiol, estriol, estrone or ethinylestradiol at levels above LOQ (30–40 ng L^{-1}) in either ozonated or non-ozonated effluent.

Ozone treatment has been reported to decrease the vitellogenin gene expression levels in male fish induced by conventional STP effluents (Cuklev et al., 2012; Gunnarsson et al., 2009). In the current study however, exposure to the ozone treated STP effluent unexpectedly resulted in higher vitellogenin gene expression levels compared with the other treatments. This might indicate that the chemical composition of the effluent has become more estrogenic due to ozonation by-products, an outcome which has been illustrated in earlier studies. Ozonation of a hospital wastewater effluent has been shown to produce chemicals which stimulate endogenous production of estrogens in fish (Maletz et al., 2013). Furthermore, ozonation of 17β-estradiol might increase its *in vitro* estrogenic activity, as indicated in laboratory studies (Bila et al., 2007). The results we present in this study may thus be associated with ozone-mediated estrogenicity specific to the presently studied STP effluent and the applied ozonation procedure.

3.3. Effects on zebrafish reproduction

The fecundity differed significantly between the treatments; fish in the ozonated STP effluent group produced twice the amount of eggs ($p = .0021$) as compared to both the tap water control and the conventional STP effluent (Table 2). The cumulative egg production per female was conclusively greater in the ozonated STP effluent group (Fig. 2). The proportion of fertilized and damaged eggs did not differ significantly between the treatments (Table 2).

The fish reproductive system is controlled by several hormonal and paracrine pathways that may potentially become disrupted by xenobiotic chemicals. Prostaglandins are synthesized in the ovary and have been implicated to induce egg production and spawning in female zebrafish (Lister and Van Der Kraak, 2008). Furthermore, prostaglandins may increase male spawning behaviors by acting as hormonal pheromones (Stacey et al., 2003). The nonsteroidal anti-inflammatory drug ibuprofen is found in wastewater influenced aquatic environment and has the capacity to impact the fish prostaglandin synthesis (Han et al., 2010; Morthorst et al., 2013). The chemical analysis in the present study did however not reveal any prostaglandin interfering APIs.

One reason for increased fecundity in fish could be explained by low concentrations of estrogenic xenobiotics, as shown in several lab studies. Exposure to ethinylestradiol in the 1 ng L^{-1} range induces egg-laying in fathead minnow (Jobling et al., 2003). Increased egg production has also been observed in gulf pipefish (*Syngnathus scovelli*) exposed to low ethinylestradiol concentrations (2 ng L^{-1}) (Rose et al., 2013). Nonylphenol exposure may induce fecundity in Japanese medaka (Nimrod and Benson, 1998) and in fathead minnow (Giesy et al., 2000). The increase in fecundity in the present study might partly be caused by exposure to low concentrations of estrogenic compounds present in the ozonated effluent water. This could indicate that ozonation, under the particular conditions of this study, created by-products more estrogenic than the parent compounds measured in the non-ozonated STP effluent.

While an increased production of eggs could potentially be a reaction to an inhospitable environment, it could also be a response to an ideal environment. Improved fecundity in fish exposed to ozonated effluents have been reported in previous literature. Egg production in Japanese medaka (*Oryzias latipes*) exposed to ozonated STP effluent in a similar setup as the present study increased slightly, yet not significant, compared to control and conventional STP treatments (Altmann et al., 2012). Increased egg production has been recorded in fathead minnow (*Pimephales promelas*) exposed to ozonated STP effluent (Ward and DeGraeve, 1978). In these studies, as well as the present study, the obtained overall quality of the ozonated effluent treatment is possibly the most optimal for the fish, thereby resulting in a normal reproductive output. The tap water and non-ozonated STP effluent may represent sub-optimal conditions, thereby resulting in lower fecundity compared with the ozonated STP effluent. Thus, the reproductive outcome from fish exposed to ozonated effluent might not display an increase in reproductive success but rather a normal level of reproduction.

Histological assessment of the ovarian sections (Fig. 3) revealed that the ozonated STP effluent treatment group contained a larger proportion of vitellogenic stage oocytes than the tap water control (Fig. 4). The proportion was also slightly larger in the conventional STP effluent treatment group compared to the tap water control group, albeit not significant (Fig. 4). The hormonal basis of oocyte maturation in female fish is related to LH, which stimulates maturation inducing hormone in the ovary (Nagahama and Yamashita, 2008). The results of the present study shows that egg maturation was amplified in the ozonated STP, perhaps due to endocrine active ozonation by-products or just optimal water conditions. There were no signs of intersex in male fish (data not

Table 2
Summary of egg production data assembled during the last 7 days of the 21 day zebrafish exposure. A spawning was considered successful if eggs were present after the 30 min spawning period after lights on (9.00 am–9.30 am). The numbers of fertilized and damaged eggs is presented as treatment replicate means ± standard deviation (n = 3) of quantity (n) and proportion (%) of the eggs collected.

Treatment	Tank replicate	Successful spawnings (out of 7)	Fertilized eggs produced (per female, mean ± sd)		Damaged eggs (per female, mean ± sd)	
			(n)	(%)	(n)	(%)
Tap water control	1	5	34.4 ± 13.8	89.8 ± 5.0	5.3 ± 5.4	8.6 ± 4.3
	2	5				
	3	6				
STP effluent	1	5	38.5 ± 1.9	94.2 ± 6.9	1.2 ± 0.8	4.9 ± 5.3
	2	3				
	3	5				
Ozonated STP effluent	1	5	77.5 ± 7.5 *	83.2 ± 14.3	8.9 ± 2.3	15.3 ± 14.5
	2	4				
	3	5				

* Treatment difference (ANOVA $p = .0021$, Tukey's HSD $p < .05$).

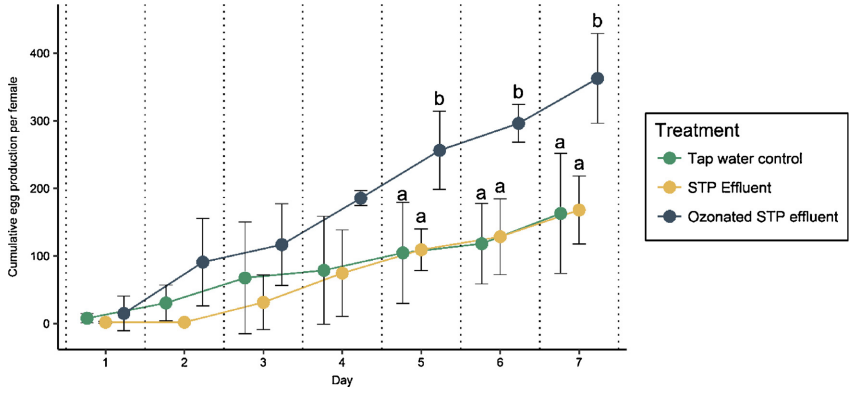

Fig. 2. Total cumulative number of fertilized eggs produced per female and day during the last 7 days of the 21 day exposure (n = 3, mean ± sd). Different letters indicate significant differences between the treatments (pair-wise Tukey's HSD $p < 0.05$) at day five, six and seven (ANOVA p = .0087).

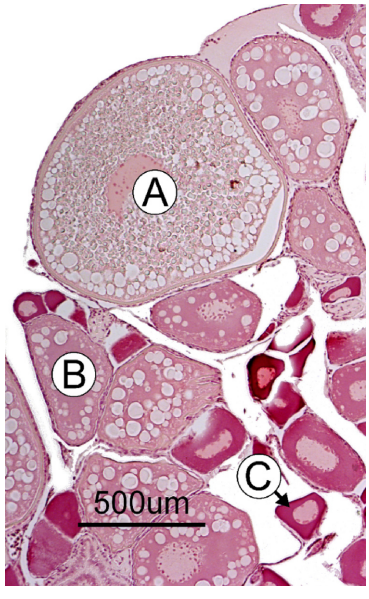

Fig. 3. Microphotograph of a zebrafish ovarian section exemplifying a vitellogenic (A), cortical alveolar (B) and primary oocyte (C).

shown). All the males had comparable amounts of spermatozoa in their seminiferous tubules.

3.4. Larvae locomotor activity, mortality and malformations

Embryos collected from the fecundity assessment stage were allowed to develop for 6 dpf in its corresponding aquarium water before locomotion testing. There were no statistically significant differences in mortalities or malformations between the treatment groups (Table 3). The larvae locomotion test did not reveal any differences in activity between the treatment groups (Fig. 5). Larvae locomotor activity has been used as a sublethal endpoint for assessing developmental toxicology in exposed fish progeny (MacPhail et al., 2009). Several different classes of suspected neurotoxic chemicals has been screened for neurobehavioral effects using this approach (Ellis et al., 2012; Ulhaq et al., 2013). In the present study, such effects were not detected.

3.5. Swimming activity in adult zebrafish

Fish exposed to the ozonated STP effluent were significantly less active ($p < .0001$) during the first minute of the trial (Fig. 6). Over time the activity of fish in the ozonated treatment increased to that of the other treatment groups. There were no evident difference in activity between the sexes (p = .0656) and therefore the statistical model was run including both males and females.

A reduced swimming activity in zebrafish is generally associated with an increased stress phenotype (Kalueff et al., 2013). The stress response is important for coping and adapting to the environment but may lead to a pathological outcome in the long run (Bonga, 1997). The serotonergic system is associated with stress in fish where stressed individuals have increased levels of serotonin in the brain (Winberg et al., 1992). Serotonin modulates the hypothalamus-pituitary-interrenal axis, or the 'stress-axis', in fish. Serotonin also stimulates the reproductive neuroendocrine axis in fish by increasing the release of gonadotropins from the pituitary gland, which controls gonad maturation and egg release (Somoza et al., 1988). Whether endocrine disruption of the serotonergic system is influencing the observed fecundity and stress effects in the current study remains speculative at this point. However, recent experimental evidence supports the notion that STP effluent ozonation modulates serotonergic responses in fish. Rainbow trout injected intraperitoneally with ozonated wastewater extract has been shown to induce brain serotonin levels (Maya et al., 2017). It is difficult to extrapolate whether these effects, reported both in previous literature and in the present study, would have an adverse impact on wild fish populations.

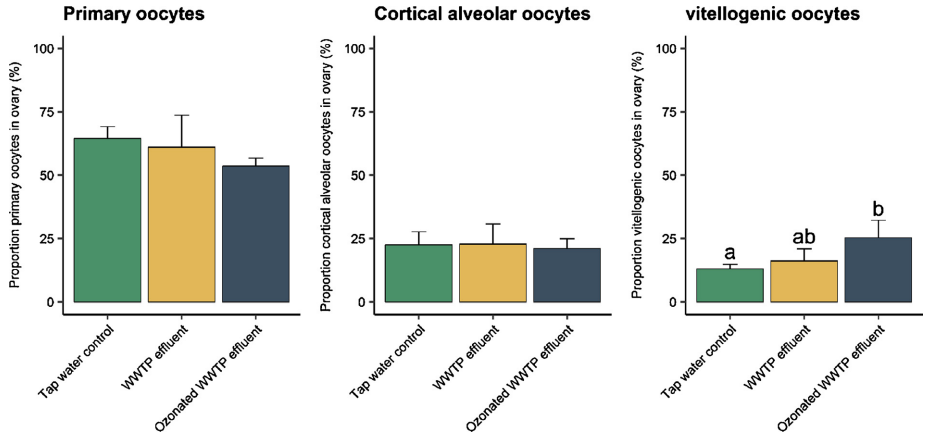

Fig. 4. Proportion of primary oocytes, cortical alveolar oocytes and vitellogenic oocytes within the ovary (n = 3, mean + sd). Different letters indicate significant differences (Tukey's HSD $p < 0.05$). Ovaries in fish exposed to the ozonated STP effluent treatment group contained more vitellogenic oocytes (C) compared to the tap water control (ANOVA p = .0424).

Table 3
Mortality at 24, 48 and 144 hpf and malformations at 144 hpf in zebrafish larvae progeny from the first successful spawning in the corresponding replicate tank (n = 3, mean ± sd).

	Mortality at 24 hpf (%)	Mortality at 48 hpf (%)	Mortality at 144 hpf (%)	Malformations at 144 hpf (% of alive)
Tap water control	0 ± 0	9.7 ± 9.6	13.9 ± 17.3	13.4 ± 16.1
STP effluent	26.1 ± 45.2	36.5 ± 36.3	50 ± 45.1	5 ± 7.1
Ozonated STP effluent	2.8 ± 4.8	5.6 ± 2.4	34.7 ± 18.8	7.9 ± 9.7

4. Conclusions

We have reported that 24 of the 105 screened pharmaceuticals were present in the conventionally treated STP effluent at concentrations above LOQ. Ozonation removed on average 77% of the concentrations of these 24 pharmaceuticals. The male hepatic gene expression of *VTG-2* was induced in fish exposed to ozonated STP effluent indicating an estrogenic effect. The estrogenicity was not of such magnitude that it negatively affected fecundity. On the contrary, zebrafish exposed to ozonated effluent exhibited a two-fold increased fecundity compared to both the tap water control and conventional STP effluent treatment groups. This ambiguity could partly be explained by either the ozonated STP effluent being the best spawning environment for the fish, or be the result of estrogenic ozonated by-products stimulating reproduction. The occurrence of ozonation by-products were however not analyzed in the ozonated STP effluent.

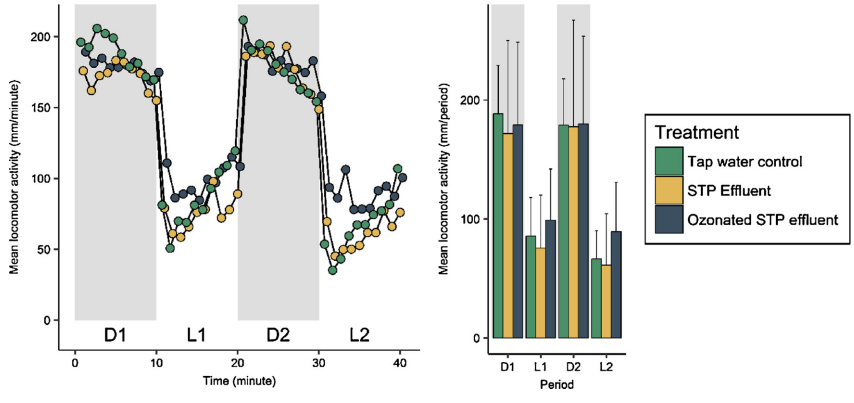

Fig. 5. Larvae locomotor activity during four alternating 10 min dark (D1 and D2) and light (L1 and L2) periods. The left panel shows locomotor activity per minute (n = 3, mean), the right panel shows locomotor activity per period (n = 3, mean + SD). The locomotor activity response to alternating light and dark periods was not different between the treatment groups.

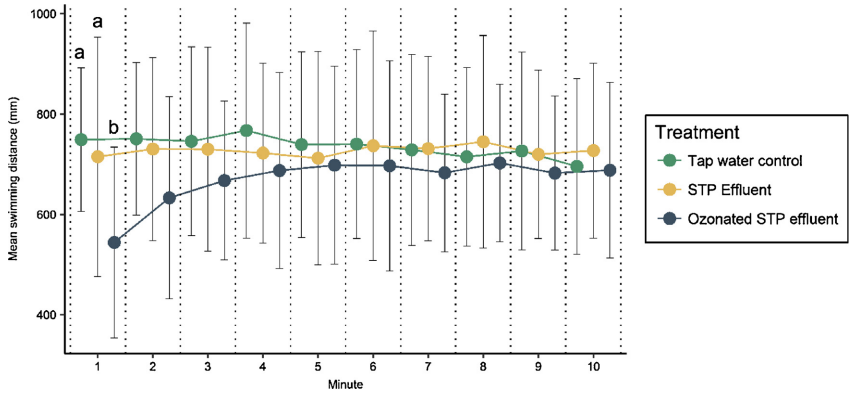

Fig. 6. Swimming activity in adult zebrafish during 10 min after introduction to a novel vessel (n = 3, mean ± sd). Different letters indicate significant differences between the treatments (pair-wise Tukey's HSD $p < 0.05$) during the first minute (ANOVA $p < 0.0001$).

The results from the present study highlight the importance of monitoring the implementation of full-scale STP effluent ozonation by both chemical and biological effect screening. Future research is needed for uncovering the potencies and identities of ozonated by-products that cause *in vivo* responses in fish.

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Effects of Ozonated Wastewater Effluent on Reproduction and Behavioral Endpoints in Zebrafish (*Danio rerio*)

Supporting information

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Table 1. List of the 105 analyzed APIs.

Pharmaceutical substance name		
Alfuzosin	Flecainide	Sertraline
Alprazolam	Fluconazole	Sulfamethoxazol
Amiodarone	Flunitrazepam	Tamoxifen
Amytriptyline	Fluoxetine	Telmisartan
Atenolol	Flupentixol	Terbutaline
Atorvastatin	Fluphenazine	Tetracycline
Atracurium	Flutamide	Tramadol
Azelastine	Glibenclamide	Trihexyphenidyl
Biperiden	Glimepiride	Trimethoprim
Bisoprolol	Haloperidol	Venlafaxine
Bromocriptine	Hydroxyzine	Verapamil
Budesonide	Ibuprofen	Zolpidem
Buprenorphine	Irbesartan	
Bupropion	Ketoconazole	
Carbamazepin	Ketoprofen	
Chlorpromazine	Levomepromazine	
Chlorprothixene	Levonorgestrel	
Cilazapril	Loperamide	
Ciprofloxacin	Maprotiline	
Citalopram	Meclozine	
Clarithromycine	Medroxyprogesterone	
Clemastine	Megestrol	
Clindamycin	Memantine	
Clomipramine	Metoprolol	
Clonazepam	Mianserin	
Clotrimazol	Miconazole	
Codeine	Mirtazapine	
Cyproheptadine	Naloxone	
Desloratidin	Naproxen	
Diclofenac	Nefazodone	
Dicycloverine	Norfloxacin	
Dihydroergotamine	Ofloxacin/levofloxacin	
Diltiazem	Orphenadrine	
Diphenhydramine	Oxazepam	
Donepezil	Oxytetracycline	
Duloxetine	Paracetamol	
Eprosartan	Paroxetine	
Estradiol	Perphenazine	
Estriol	Pizotifen	
Estrone	Progesterone	
Ethinyl estradiol	Promethazine	
Etonogestrel	Ranitadine	
Fenofibrate	Repaglinide	
Fentanyl	Risperidone	
Fexofenadine	Rosuvastatin	
Finasteride	Roxithromycine	

Table 2. Oligonucleotide sequences (5' to 3') of the primers used in the present study.

Transcript		Oligonucleotide sequence	Accession number	Reference
ACBT	Forward	ACAGGGAAAAGATGACACAGATCA	AF025305	Lister and Van Der Kraak, 2008
	Reverse	CAGCCTGGATGGCAACGTA		
CYP1A1	Forward	CTGGACGAAAACTCCAACCTG	NM_131879	Lister et al., 2009
	Reverse	GATAGTGTCGAAACCGGCTCC		
ERα	Forward	AAACACAGTCGGCCCTACAC	AJ414566	Arukwe et al., 2008
	Reverse	GCCAAGAGCTCTCCAACAAC		
VTG-2	Forward	GGTGACTGGAAGATCCAAG	AY729645	Jin et al., 2008
	Reverse	TCATGCGGCATTGGCTGG		

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Embryotoxicity of ozonated diclofenac, carbamazepine, and oxazepam in zebrafish (*Danio rerio*)

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HIGHLIGHTS

- First time evaluation of *Danio rerio* embryo toxicity of ozonated pharmaceuticals.
- Ozonation of carbamazepine increased the toxicity in all tested endpoints.
- Three analyzed carbamazepine ozonation by-products were formed.
- Diclofenac toxicity was completely eliminated by ozonation.
- Ozonated oxazepam exposure elicited swimming behavioral changes in 6 day old larvae.

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ABSTRACT

Pharmaceutical residues are polluting the surface water environments worldwide. Sewage and wastewater treatment, therefore, needs to be improved in order to remove pharmaceutical residues from the effluent. One such treatment improvement is effluent ozonation. Even though ozonation has proven to be very efficient in reducing pharmaceutical parent compound concentrations in wastewater effluents, much remains unclear regarding potentially toxic ozonation by-product (OBP) formation. In this study, we sought to elucidate the aquatic toxicity of ozonated pharmaceuticals in zebrafish (*Danio rerio*) embryos in a static 144 h post fertilization (hpf) fish embryotoxicity (ZFET) assay. Three pharmaceuticals commonly detected in wastewater effluents, i.e. carbamazepine, diclofenac, and oxazepam, were selected for testing. Toxicity was assessed before and after 1 min ozonation (0.053 mg L^{-1} peak O_3 concentration) and 10 min ozonation (0.147 mg L^{-1} peak O_3 concentration). Chemical analysis showed that carbamazepine and diclofenac were largely removed by ozone ($90 \pm 11\%$ and $97 \pm 3.8\%$), whereas oxazepam was removed to a lesser extent ($19 \pm 5.7\%$). The ZFET assay revealed diverging toxicities. Diclofenac embryotoxicity decreased with increasing ozonation. Oxazepam did not cause embryotoxicity in the ZFET assay either pre- or post ozonation, but larvae swimming activity was affected at 144 hpf. Carbamazepine embryotoxicity, on the other hand, increased with increasing ozonation. Chemical analysis showed the formation of two OBPs (carbamazepine-10,11-epoxide and 10,11-dihydrocarbamazepine), possibly explaining the increased embryotoxicity. The results of this study highlight the importance of new chemical and toxicological knowledge regarding the formation of OBPs in post-ozonated effluents.

1. Introduction

Pharmaceutical residue pollution is a considerable challenge regarding water quality and ecological status of the aquatic environment (Fent et al., 2006). A major pollution pathway into the aquatic environment is domestic effluent from municipal sewage treatment plants (STPs). STPs are often not capable to efficiently remove organic micropollutants such as pharmaceutical residues from the effluent (Luo et al., 2014). Thus, STPs discharge

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pharmaceutical contamination into the surface water environments, elevating the concerns of adverse effects in aquatic biota (aus der Beek et al., 2016; Hughes et al., 2013; Loos et al., 2013). Conventionally treated municipal STP effluents often contain the anti-inflammatory drug diclofenac, the anti-epileptic carbamazepine and the anxiolytic drug oxazepam in the range of several hundred ng L^{-1} (e.g. Lindberg et al., 2014; Pohl et al., 2018). Some pharmaceuticals are refractory in nature and may have bioaccumulative properties in the aquatic environment (Grabicova et al., 2017). Since STP effluents release pharmaceutical residues into the recipient continuously, the resulting pollution can be considered to be pseudo-persistent. A recent study has shown that carbamazepine has been detected in all parts of the Baltic Sea (Björlenius et al., 2018). Effects on wildlife due to pharmaceutical residues in the aquatic environment are not yet well understood, but pharmaceuticals made for human and veterinary usage can potentially affect non-target organisms such as fish (Gunnarsson et al., 2008). Laboratory studies have shown how carbamazepine (10 μg L^{-1}) produces xeno-estrogenic effects in fish (Yan et al., 2018). Oxazepam (200 μg L^{-1}) affects wild fish behaviors implicating adverse effects on an ecological scale (Brodin et al., 2013; Klaminder et al., 2016). Other examples include diclofenac (4.6 μg L^{-1}) adversely affecting three-spined stickleback (*Gasterosteus aculeatus*) kidney function (Näslund et al., 2017). As a consequence, regulations are being put in place to reduce the load of pharmaceutical contamination in the aquatic environment. Diclofenac has, for example, been added to the 2013/39/EU directive watch list of priority substances (European Union, 2015).

STP effluent ozonation is being implemented in Sweden in order to alleviate pharmaceutical pollution (Baresel et al., 2016). Ozone has the capacity to reduce the pharmaceutical load of STP effluents (Prasse et al., 2015). Previous ecotoxicological evaluations have shown beneficial effects of whole-effluent ozonation (e.g. Beijer et al., 2017; Cuklev et al., 2012; Lundström et al., 2010). Some studies, however, have suggested increased toxicity in fish following effluent ozonation (e.g. Costa et al., 2014; Giebner et al., 2016). While ozonation has proven to be very efficient in reducing pharmaceutical parent compound concentrations in STP effluents, the formation of potentially toxic ozonation by-products (OBPs) remains insufficiently studied. Thus, it is important to explore if and how the ozonation treatment of STP effluents modulates the biological effect in aquatic organisms exposed to the STP effluent due to suspected OBP toxicity.

The present study aimed to determine the toxicity of untreated and ozonated carbamazepine, diclofenac, and oxazepam. These three particular pharmaceuticals were selected since they are commonly detected in sewage effluents and aquatic environments in the EU (Loos et al., 2013). The toxicological evaluation was performed using the zebrafish (*Danio rerio*) embryo toxicity assay (ZFET), which represents a robust whole organism aquatic toxicity test for detecting effects on lethal and sublethal endpoints (Hill et al., 2005). Furthermore, it can be combined with a behavioral (larvae swimming activity) assay in order to detect neurobehavioral effects due to developmental chemical exposure (Selderslaghs et al., 2013). In addition, the ozonated extracts were screened for four putative transformation products of carbamazepine to explain observed toxicity.

2. Materials & methods

2.1. Chemicals

Carbamazepine (CAS number 298-46-4, purity ≥ 98%), diclofenac sodium salt (CAS number 15307-79-6, purity ≥ 98%) and oxazepam (CAS number 604-75-1, purity ≥ 98%) acquired from Sigma-Aldrich (Sweden) were used in the study. Carbamazepine and diclofenac were directly diluted into carbon filtered tap water (control water) at maximum nominal concentrations and then serially diluted 1:2 (100, 50, and 25 mg L^{-1} for carbamazepine and 15, 7.5, and 3.8 mg L^{-1} for diclofenac). Oxazepam was first diluted in dimethyl sulfoxide (DMSO, purchased from Sigma-Aldrich (Sweden), CAS number 67-68-5, purity ≥ 99%) at 100 mg mL^{-1}, serially diluted in DMSO 1:10 and then mixed in control water to reach final nominal test concentrations of 10, 1 and 0.1 mg L^{-1} (0.01% DMSO). Metabolite standards of carbamazepine: carbamazepine-10,11-epoxide (Cbz-Ep, CAS number 36507-30-9, purity ≥ 98%), 10,11-dihydro-10-hydroxycarbamazepine (DiOH-Cbz, CAS Number 29331-92-8, purity ≥ 99%), 10,11-dihydrocarbamazepine (Di-Cbz, CAS Number 3564-73-6, purity ≥ 99%), and oxcarbazepine (Ox-Cz, CAS Number 28721-07-5, purity ≥ 97.5%) were purchased from Sigma-Aldrich (Sweden). Isotope-labeled internal standards of oxazepam (D5) was acquired from lipomed AG (Sweden) and diclofenac ($^{13}C_6$) was purchased from Sigma-Aldrich (Sweden). Ultrapure water was produced by a Milli-Q Advantage Ultrapure Water purification system and filtered through a 0.22 μm Millipak Express membrane and an LC-Pak® polishing unit (Merk Millipore, Billercia, MA). Methanol, acetonitrile and ammonium acetate were all of high-performance liquid chromatography (HPLC)-grade and purchased from Sigma Aldrich (St. Luis, MO, USA).

2.2. Experimental ozonation set-up

Ozonation was performed using an ozone generator (AB Aqua Medic GMBH, Bissendorf, Germany) with a maximum ozone generation capacity of 100 mg O_3 h^{-1} set up within a fume-hood. The ozone was administered to the test solutions (v = 0.25 L, 25 °C) by diffusion stone in Erlenmeyer flasks. The set-up performance was evaluated before pharmaceutical ozonation and zebrafish embryotoxicity tests. The ozone concentration was 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). Ozone concentrations in control water were determined over the course of 135 min continuous ozonation (Supplementary Data Fig. S1). Based on the set-up evaluation, two doses of ozonation were selected for pharmaceutical ozone treatment; 1 min ozonation (peak ozone concentration of 0.053 mg ozone L^{-1}), and 10 min ozonation (peak ozone concentration of 0.147 mg ozone L^{-1}).

Each pharmaceutical test solution and control water was ozonated either 0, 1 or 10 min directly prior to the ZFET assays (see section 2.4). The pharmaceutical concentrations were determined prior to ozonation by concentration-response range screening using the same ZFET methodology. Dissolved oxygen, conductivity and pH were measured in all solutions directly post ozonation. These water parameters did not differ between untreated nor ozonated control water or pharmaceutical solutions (pH 8.20 ± 0.065, conductivity 440 ± 9.3 μS cm^{-1}, dissolved oxygen 94 ± 0.83%, Supplementary Data Table S1). Samples (2 mL, one sample per test solution) were collected and stored in glass vials at −18 °C for chemical analysis.

2.3. Chemical analysis

The chemical analysis method was based on a previously published study (Koba et al., 2018). The samples were filtered using a regenerated cellulose syringe filter (0.22 μm pores). One milliliter of the filtered extract was placed in an autosampler vial with 10 ng of the isotope-labeled internal standards of oxazepam 80 (D5) and diclofenac ($^{13}C_6$) per aliquot of sample. The samples were analyzed using liquid chromatography tandem-mass spectrometry (LC-MS/

MS) with an LC system from Thermo Fisher Scientific, San Jose, CA, USA and a triple-stage quadrupole MS/MS TSQ Quantiva (Thermo Fisher Scientific). 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. Injection volume was 10 μL for all samples. A heated electrospray ionization (H-ESI) 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 (SRM) transitions were monitored for all analytes. Data were evaluated using TraceFinder™ 3.3 software (Thermo Fisher).

2.4. Zebrafish embryotoxicity tests

Groups of adult zebrafish kept at 12/12 h light/dark conditions were placed in stainless steel mesh spawning cages in the morning at lights on (9:00–9:30 a.m.). Eggs were collected and assessed for fertilization success where the spawning group with the highest amount of fertilized eggs (>90%) was selected for testing. Each treatment (control, low, medium and high pharmaceutical concentration with either 0, 1 or 10 min ozonation, see Table 1) consisted of 16 embryo replicates distributed in two 96-well plates. The embryos were placed in individual wells including 250 μL treatment water. The well plates were sealed with Parafilm M (Bemis NA, United States) for the duration of the exposure. The eggs were exposed statically (i.e. test solutions were not renewed over time) from fertilization until 144 h post fertilization (hpf).

Lethal (e.g. coagulation) and sublethal (e.g. incidence of yolk sac and/or pericardial edemas etc.) endpoints were recorded at 24, 48 and 144 hpf. Representative malformation types are displayed in Supplementary Data Fig. S5. At 48 hpf the heart rate was assessed in the embryos by stereo microscopy. Time-lap photographing was performed between ~50 and 144 hpf taking one picture per hour of the whole plates (Canon EOS 500D). Hatching time was determined for each embryo as the time for the photograph where the embryo first was observed as hatched. At 144 hpf, larvae locomotion (movement) in response to alternating dark and light periods was tracked using the ZebraBox tracking system and ZebraLab software (ViewPoint, France). Each 96-well plate was placed in the Zebrabox recording chamber between 1:00 and 3:00 p.m. After a preliminary 15 min acclimatization in darkness, three interchanging light and dark periods (10 min each) were executed. The distance moved by each larva (exceeding 3 mm s^{-1}) was thus simultaneously recorded and aggregated in 10 s intervals over the course of 45 min. The individual larvae total locomotion was summed into three response variables; swimming distance/min, total swimming distance during darkness, and total swimming distance during light. Data from affected embryos (malformed/dead) were excluded from the locomotion test in order not to confound the analysis.

2.5. Statistics

The RStudio version 1.0.153 interface running R software version 3.3.2 was used for data analysis and visualization (R Core team, 2016; RStudio Team, 2016). Continuous parameters (e.g. heart rate) were analyzed by one-way ANOVA followed by Dunnett's post hoc test. The incidence of mortality and malformations were combined into one binary response (affected/not affected) and analyzed by Bonferroni adjusted Fisher's exact test. The level of significance (alpha) was set at 0.05. Figures were made using the ggplot2 R package (Wickham, 2009).

3. Results & discussion

3.1. Ozone removal efficiency of carbamazepine, diclofenac, and oxazepam

The nominal concentrations for carbamazepine and oxazepam corresponded with the measured concentrations on average by 59% and 81% respectively (Table 1). These deviations were probably due to issues of solubility and precipitation in water (for carbamazepine) and DMSO (for oxazepam). The measured concentrations of diclofenac were in good agreement with nominal concentrations (Table 1). Ozonation for 1 min removed 20 ± 3.4% carbamazepine, 25 ± 16% diclofenac, and 2.0 ± 4.4% oxazepam (Fig. 1). Increasing ozone dosage time improved removal of the parent compound in all three tested pharmaceuticals. Ozonation for 10 min removed 90 ± 11% carbamazepine, 97 ± 3.8% diclofenac, and 19 ± 5.7% oxazepam. As a comparison, ozonation at an STP equipped with a full-scale ozonation plant removed 97% carbamazepine, 99% diclofenac and 42% oxazepam (Pohl et al., 2018), suggesting that the experimental setup served as a reasonable model for larger ozonation facilities.

3.2. Carbamazepine embryotoxicity before and after ozone treatment

To evaluate the effect of ozone treatment on carbamazepine, diclofenac, and oxazepam embryotoxicity, lethal and sublethal effects were recorded before and after ozonation. Carbamazepine exposure resulted in embryotoxicity at 144 hpf in the 50 mg L^{-1} and 100 mg L^{-1} treatment groups with 88% and 100% affected embryos (dead and malformed combined) respectively (Bonferroni adjusted Fisher's exact test $p < 0.001$, Fig. 3a). The 50 mg L^{-1} group consisted of 8 malformed embryos (side-lying with or without scoliosis) out

Table 1
Nominal and measured treatment concentrations (mg L^{-1}, NA = not analyzed, LOQ = limit of quantification).

Chemical	Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Measured in % of nominal concentration	LOQ (mg L^{-1})
Carbamazepine	0	<LOQ	100	$3.3e^{-6}$
	25	17	68	
	50	30	60	
	100	50	50	
Diclofenac	0	<LOQ	100	$4.0e^{-6}$
	3.8	NA	NA	
	7.5	7.4	99	
	15	15	100	
Oxazepam	0	<LOQ	100	$3.0e^{-6}$
	0.10	0.09	90	
	1.0	0.84	84	
	10	7.0	70	

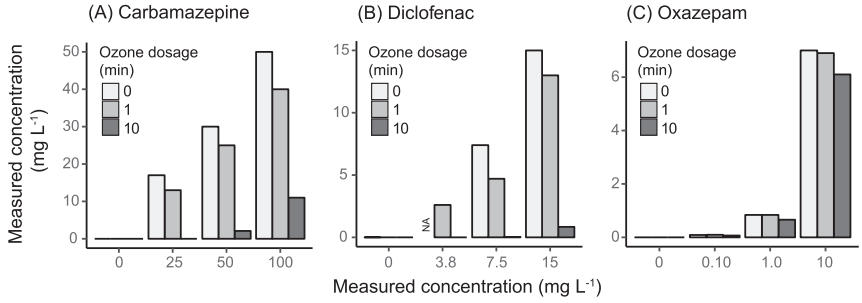

Fig. 1. Ozone removal efficiency of (A) carbamazepine, (B) diclofenac and (C) oxazepam after 0, 1 and 10-min ozonation. The lowest concentration of diclofenac (3.8 mg L^{-1} nominal concentration) was not analyzed (NA).

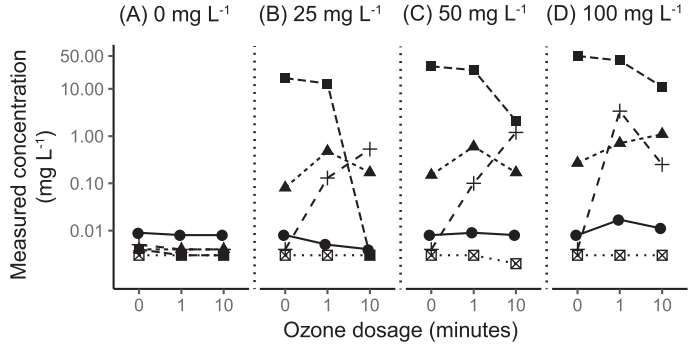

Fig. 2. Measured concentrations (mg L^{-1}) of carbamazepine (■) and suspected carbamazepine OBPs carbamazepine-10,11-epoxide (+); 10,11-dihydrocarbamazepine (▲); 10,11-dihydro-10-hydroxycarbamazepine (●), and oxcarbazepine (⊠) in the different test solutions (0, 25, 50, 100 mg L^{-1} nominal concentrations) in dependence of the ozonation dosage.

of in total 16 while 14 out of in total 16 embryos were dead in the 100 mg L^{-1} treatment at 144 hpf (Supplementary Data Table S2). Furthermore, carbamazepine exposure significantly reduced the heart rate (ANOVA $p = 0.005364$, Dunnett's $p < 0.01$) at 48 hpf in the highest treatment group (Fig. 4a). Hatching time showed a trend of delay with increasing carbamazepine concentration, and in the highest treatment group (100 mg L^{-1}) all embryos were dead prior to hatching (Fig. 5a). The pre-hatching mortalities were preceded by the incidence of pericardial and yolk-sac edemas recorded at 48 hpf. The swimming activity assay at 144 hpf showed a reduction in swimming distance (in the 50 mg L^{-1} treatment group) during both light and dark periods, albeit non-significant likely due to poor replication ($n = 2$ unaffected larvae, Supplementary Data Fig. S2).

Carbamazepine toxicity during zebrafish embryo development has been investigated in previous studies, with results in line with the present study. Beker van Woudenberg et al. (2014) reported that the most sensitive endpoint observed in zebrafish embryos was a delayed onset of hatching (72 hpf, EC_{50} 45.5 mg L^{-1}) and pericardial edema (96 hpf, EC_{50} 52 mg L^{-1}). Furthermore, larvae exposed to 43 and 57 mg L^{-1} carbamazepine were hypoactive during dark periods (Beker van Woudenberg et al., 2014). Exposure to carbamazepine (1 μg L^{-1}) has also been reported to increase body length and cause behavioral effects in zebrafish embryos (Qiang et al., 2016). Besides early development toxicity in zebrafish, carbamazepine has also been shown to cause reproductive toxicity in exposed (100 μg L^{-1}) sexually mature Chinese rare minnows (*Gobiocypris rarus*; Yan et al. (2018)).

Ozonation of carbamazepine increased toxicity in all endpoints in the present study. The lowest observed effect concentration (LOEC) of carbamazepine based on percent affected at 144 hpf was reduced from the medium concentration (50 mg L^{-1}) to the lowest (25 mg L^{-1}) following ozonation for 10 min (Fig. 3a). The increased toxicity in the lowest carbamazepine concentration ozonated 10 min was mainly manifested by side-lying (due to lack of swim bladder inflation) at 144 hpf. The sublethal endpoints heart rate (Fig. 4a) and hatching time (Fig. 5a) followed the same pattern of increased toxicity following 10 min carbamazepine ozonation. Ozonation for 10 min caused reduced heart rate in the lowest (25 mg L^{-1}) concentration treatment group (ANOVA $p = 6.79e^{-16}$, Dunnett's $p < 0.001$). The larvae swimming activity assay revealed a hypoactivity response in individuals exposed to ozonated carbamazepine (Supplementary Data Fig. S2). Altogether, these results indicate that carbamazepine ozonation produced toxic OBPs implying reduced embryo survivability. In a cell viability test (*Pseudomonas* sp. Strain KSH-1), higher toxicity was recorded after

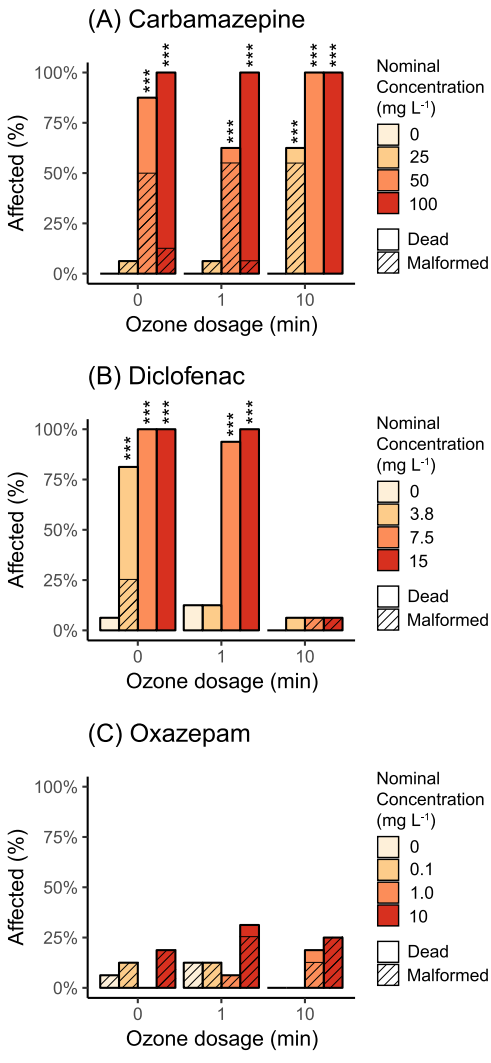

Fig. 3. Affected (% out of 16 replicates) zebrafish embryos at 144 hpf. Significant differences (***: $p < 0.001$) between treatments and control were detected for carbamazepine and diclofenac (Bonferroni adjusted Fisher's exact test).

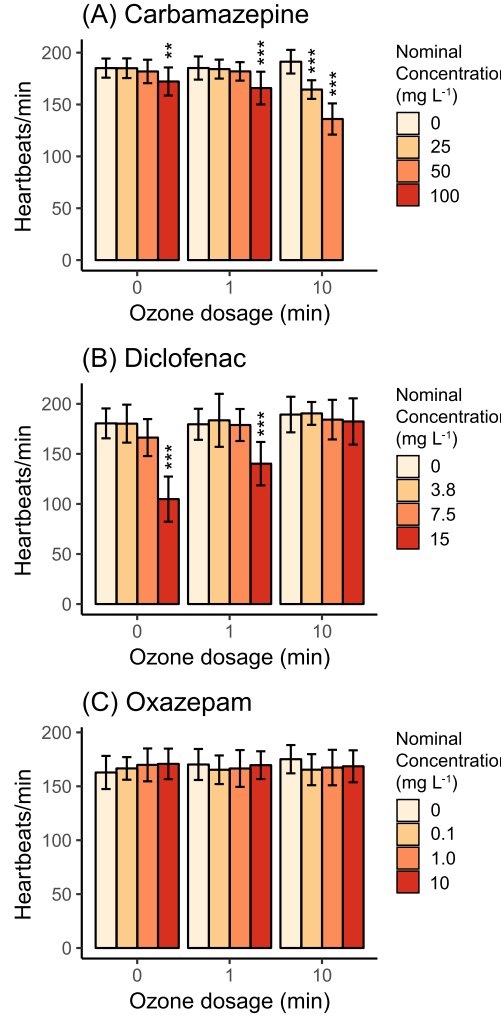

Fig. 4. Heart rate (heartbeats/minute, mean ± sd) in zebrafish embryos at 48 hpf. Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test (**: $p < 0.01$, ***: $p < 0.001$).

10 and 30 min ozonation time, which then was removed after 60 and 120 min ozonation (Dwivedi et al., 2018). Ozonated carbamazepine has also been shown to increase cytotoxicity and genotoxicity in cell based *in vitro* test systems (Han et al., 2018). OBPs, in particular, carbamazepine-10,11-epoxide, are more toxic than the parent compound (LC_{50}: 0.2 mg kg^{-1} and 1.1 mg kg^{-1}, respectively) to the macroinvertebrate *Chironomus riparius* (Heye et al., 2016). To our knowledge, there are no other studies reporting zebrafish embryotoxicity of ozonated carbamazepine to date.

3.3. Carbamazepine OBP formation

Four carbamazepine OBPs were possible to analyze being commercially available as reference standards. Cbz-Ep was detected in all ozonated carbamazepine samples, with the largest amount (3.4 mg L^{-1}) formed in the highest carbamazepine concentration ozonated for 1 min (Fig. 2). It is possible that Cbz-Ep was one major driver of increased toxicity of ozonated carbamazepine in the present study, since its concentration increased by ozone treatment (Fig. 2). The main metabolic pathway of carbamazepine in the human liver starts with the CYP3A4-mediated formation of

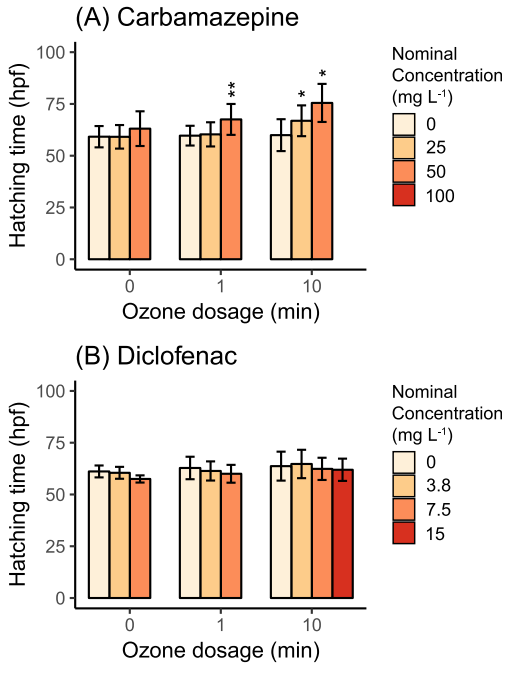

Fig. 5. Hatching time (hpf, mean ± sd) of zebrafish embryos exposed to (a) carbamazepine and (b) diclofenac (oxazepam hatching time was not recorded due to technical issues). Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test (*: $p < 0.05$, **: $p < 0.01$).

Cbz-Ep, the main therapeutically active carbamazepine metabolite (Breton et al., 2005). Cbz-Ep has also been shown to be formed in adult wild Rio de la Plata onesided livebearer (*Jenynsia multidentata*) exposed to carbamazepine, illustrating the concern of biotransformation and potential biological effects occurring in non-target species (Valdés et al., 2016).

The second most abundantly detected carbamazepine OBP in the present study was Di-Cbz. All carbamazepine treatment samples, ozonated and not ozonated, contained concentrations of Di-Cbz exceeding LOQ (Fig. 2). Concentrations of Di-Cbz in non-ozonated samples increased with increasing concentration of carbamazepine; 0.081 mg L^{-1} in the lowest concentration (Fig. 2b), 0.15 mg L^{-1} in the medium concentration (Fig. 2c), and 0.27 mg L^{-1} in the highest concentration (Fig. 2d). This could implicate the action of some metabolizing process independent of ozonation. The carbamazepine metabolite concentration has been shown to increase in some cases following treatment in conventional sewage treatment plants, presumably by biotic and abiotic processes (Leclercq et al., 2009). In the present study, ozonation for 1 min further increased concentrations of Di-Cbz. Prolonging ozonation dosage to 10 min did, however, decrease concentrations of Di-Cbz in the lowest and medium carbamazepine concentrations. Meanwhile, its concentration in the highest carbamazepine concentration continued to increase, capping at 1.1 mg L^{-1}. This may partly be explained by the chemical stability of Di-Cbz or the possibility that the higher ozone dosage was enough to further eliminate Di-Cbz in the two lower carbamazepine concentrations, but not the highest.

DiOH-Cbz was only detected (0.017 mg L^{-1}) in the highest carbamazepine concentration ozonated for 1 min (Fig. 2). DiOH-Cbz is a carbamazepine metabolite mainly formed by hepatic metabolism of Cbz-Ep and Ox-Cz (Breton et al., 2005). Ox-Cz was not detected in any sample, and thus likely not formed by carbamazepine ozonation.

3.4. Diclofenac embryotoxicity before and after ozone treatment

Diclofenac exposure (7.5 and 15 mg L^{-1} nominal concentrations) caused pericardial- and yolk-sac edemas, as well as restricted systemic circulation, at 48 hpf (Bonferroni adjusted Fisher's exact test $p < 0.001$). Furthermore, 15 mg L^{-1} exposure reduced heart rate (ANOVA $p = 3.081e^{-16}$, Dunnett's $p < 0.001$) by ~50% (Fig. 4b). These responses subsequently gave rise to 100% mortalities at 144 hpf (Fig. 3b). The lowest concentration treatment group (3.8 mg L^{-1}) did not exhibit a significant proportion of affected (3 malformed and 1 dead embryo, 25% affected) at 48 hpf, yet caused 81% affected embryos (Bonferroni adjusted Fisher's exact test $p < 0.001$) at 144 hpf (Fig. 3b). Hatching was not delayed by diclofenac exposure in the two lower exposure groups but the 15 mg L^{-1} treatment group failed to hatch due to 100% pre-hatch mortalities (Fig. 5b). No behavioral effects were detected for diclofenac in the swimming activity assay (Supplementary Data Fig. S3). Diclofenac exposure at much lower concentrations (5 μg L^{-1}) has previously been shown to delay hatching time, reduce swimming velocity and distance during light conditions, and reduce spontaneous coiling motions in zebrafish embryos (Xia et al., 2017).

Diclofenac toxicity was conclusively reduced following ozonation. Non-ozonated diclofenac toxicity was predominately manifested by pericardial edemas and reduced heart rate at 48 hpf, which was abolished completely after 10 min ozonation (Fig. 3b, Supplementary Data Table S2). The percentage number of affected embryos exposed to the lowest diclofenac concentration (3.8 mg L^{-1}) at 144 hpf, which amounted to 81% in non-ozonated group, was reduced to control group level after 1 min ozonation (Fig. 3b). Diclofenac ozonated for 10 min did not produce any toxicity in any endpoint. Likewise, ozonation led to a reduction of diclofenac toxicity in a Microtox (Vibrio fischeri NRRL B-11177 strain) *in vitro* study (Coelho et al., 2009). There are, to our current knowledge, no previous studies on zebrafish embryotoxicity of diclofenac post-ozonation.

3.5. Oxazepam embryotoxicity before and after ozone treatment

Oxazepam did not cause toxic responses in any recorded lethal or sublethal endpoint in the present study except for larvae locomotion. Ozonation of the highest oxazepam concentration (10 mg L^{-1}) at 10 min caused hypoactivity during dark conditions (ANOVA $p = 0.006535$, Dunnett's $p < 0.05$) in larvae (Fig. 6c). This concentration is much higher than concentrations known to produce behavioral effects (increased boldness) in wild perch (*Perca fluviatilis*; Brodin et al., 2013). Oxazepam is a $GABA_A$ receptor activator, an anxiolytic benzodiazepine which gives rise to GABAergic behavioral modifications. Since larvae activity is induced by dark periods (an anxiety-like response) in the larvae swimming activity assay, the hypoactivity illustrated in this and other studies may be explained by an anxiolytic mode of action (Ali et al., 2011; MacPhail et al., 2009). One possibility of this response could be the formation of OBPs potentiating the anxiolytic properties of oxazepam in the exposed zebrafish embryos. Oxazepam exposure (10 mg L^{-1}) of larval Japanese medaka (*Oryzias latipes*) decreases swimming activity during dark periods (Chiffre et al., 2016). The zebrafish embryo may be less sensitive than the Japanese medaka larvae in this respect which could be a matter of species or life stage differences.

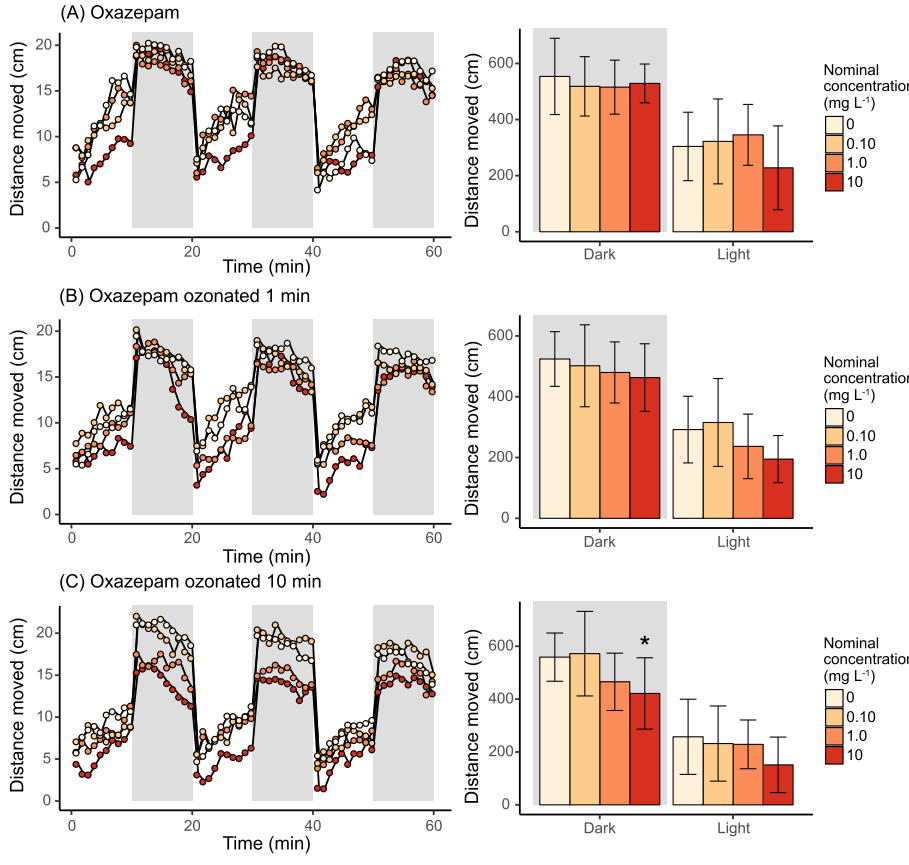

Fig. 6. Swimming activity (distance moved over time) in zebrafish embryos exposed to oxazepam ozonated 0 min (a), 1 min (b) and 10 min (c). The left panel shows total distance moved per minute (mean) and the right panel shows mean total distance moved during dark and light conditions (mean ± sd). Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test (*: $p < 0.05$), where total distance moved during darkness and light for each larva was considered an individual replicate.

The present study indicates that ozonation of oxazepam at high concentrations (10 mg L^{-1}) has the potential to produce effects on larval fish swimming behavior, which could potentially lead to effects on subsequent survivability due to impaired escape response.

3.6. Ozonation as an additional sewage treatment step

The development of safe and effective novel sewage treatment technologies relies on mitigating any toxicity arising in the post-treated effluent. A zebrafish embryotoxicity test, similar to the one conducted in the present study, has previously been used to show increasing toxicity of STP effluent following ozonation (Wigh et al., 2015). Ozone can produce aldehydic compounds in sewage effluents, being acutely toxic to early life-stage fish (Yan et al., 2014). The highly toxic compound bromate is also formed in wastewater following ozonation which poses a challenge (Soltermann et al., 2017). Likewise, we have observed that pharmaceuticals – in particular carbamazepine, may become more toxic to zebrafish embryos after ozonation. However, the pharmaceutical concentrations used as treatments in the current study were around five-fold higher than normally found in environmental matrices. Yet chronic exposure at lower, closer to environmentally relevant, concentrations of e.g. diclofenac can ultimately give rise to adverse effects in fish (Mehinto et al., 2010). Therefore, the increase of toxicity from formed carbamazepine OBPs, and oxazepam should warrant caution.

The present study shows that pharmaceutical OBPs are important to control for in post-ozonated effluents. Post-ozonation filters have been suggested as a method for removing OBPs and thereby preventing toxicity (Stalter et al., 2010). However, since carbamazepine has been shown to bio-transform into potentially more toxic metabolites in bioactive filters (Kaiser et al., 2014), a combination of ozonation and biofiltration may ultimately lead to toxicity abatement of the effluent (de Wilt et al., 2018). There are also additional treatment steps which can improve the removal efficiency of ozone, e.g. addition of hydrogen peroxide escalating OH-

radical formation (von Gunten, 2003). These ozonation 'add-ons' were not utilized in the present study and could affect toxicity outcome in further studies.

4. Conclusions

We have presented that ozonation was effective in removing carbamazepine and diclofenac from water while leaving oxazepam mostly intact. The toxicity resulting from the ozone treatment was reduced for diclofenac but increased for carbamazepine and oxazepam. The toxicity increase may be explained by OBP formation in the case of carbamazepine, where three screened carbamazepine OBPs where detected in post-ozonation samples. Oxazepam ozonation gave rise to behavioral effects possibly explained by increased bioavailability or potency. Further investigations are needed to prove the cause of the increased toxicity and find the most problematic OBP in order to prevent ozone-mediated toxicity in post-ozonated effluents before release into the recipient.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.03.034.

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Supplementary data

Embryotoxicity of ozonated diclofenac, carbamazepine, and oxazepam in zebrafish (*Danio rerio*)

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Table S1. Water parameters of the treatments directly post-ozonation

Chemical	Concentration	Ozone dosage	pH	Conductivity	Dissolved oxygen (%)
Carbamazepine	0	0	8.3	430	94
		1	8.2	429	94
		10	8.3	431	93
	25	0	8.2	434	94
		1	8.2	434	94
		10	8.2	435	96
	50	0	8.2	440	94
		1	8.2	435	93
		10	8.2	436	94
	100	0	8.3	442	94
		1	8.2	435	94
		10	8.1	439	94
Diclofenac	0	0	8.2	444	94
		1	8.2	444	94
		10	8.2	450	94
	3.8	0	8.3	450	94
		1	8.2	448	96
		10	8.1	451	94
	7.5	0	8.2	451	96
		1	8.2	449	94
		10	8.0	453	93
	15	0	8.2	448	94
		1	8.3	452	94
		10	8.0	455	93
Oxazepam	0	0	8.2	430	94
		1	8.2	428	94
		10	8.2	429	94
	0.1	0	8.2	427	94
		1	8.2	428	96
		10	8.2	428	94
	1.0	0	8.3	431	96
		1	8.2	430	94
		10	8.2	430	93
	10	0	8.2	429	94
		1	8.2	428	94
		10	8.2	429	94

Table S2. Lethality and malformation counts at 24 hpf, 48 hpf and 144 hpf for the exposure studies.

Chemical	Concentration	Ozone dosage	Assessed individuals (n, dead/malformed/normal)		
			24 hpf	48 hpf	144 hpf
Carbamazepine	0	0	0/0/16	0/0/16	0/0/16
		1	0/0/16	0/0/16	0/0/16
		10	0/0/16	0/0/16	0/0/16
	25	0	0/0/16	0/0/16	0/1/15
		1	0/0/16	0/0/16	0/1/15
		10	0/0/16	0/1/15	1/9/6
	50	0	2/0/14	2/0/14	6/8/2
		1	1/0/15	1/0/15	1/9/6
		10	0/0/16	0/3/13	16/0/0
	100	0	1/0/15	1/1/14	14/2/0
		1	0/0/16	0/5/11	15/1/0
		10	0/0/16	16/0/0	16/0/0
Diclofenac	0	0	1/0/15	1/0/15	1/0/15
		1	1/0/15	1/0/15	2/0/14
		10	0/0/16	0/0/16	0/0/16
	3.8	0	1/0/15	1/3/12	9/4/3
		1	1/0/15	1/1/14	2/0/14
		10	1/0/15	1/0/15	1/0/15
	7.5	0	0/0/16	0/14/2	16/0/0
		1	0/0/16	0/10/6	15/0/1
		10	0/0/16	0/0/16	0/1/15
	15	0	1/0/15	2/14/0	16/0/0
		1	0/0/16	0/16/0	16/0/0
		10	0/0/16	0/3/13	0/1/15
Oxazepam	0	0	0/0/16	0/0/16	0/1/15
		1	0/0/16	0/0/16	0/2/14
		10	0/0/16	0/0/16	0/0/16
	0.1	0	0/0/16	0/0/16	0/2/14
		1	0/0/16	0/0/16	0/2/14
		10	0/0/16	0/0/16	0/0/16
	1.0	0	0/0/16	0/0/16	0/0/16
		1	0/0/16	0/0/16	0/1/15
		10	0/0/16	0/1/15	1/2/13
	10	0	0/0/16	0/0/16	0/3/13
		1	0/0/16	0/0/16	1/4/11
		10	0/0/16	0/0/16	0/4/12

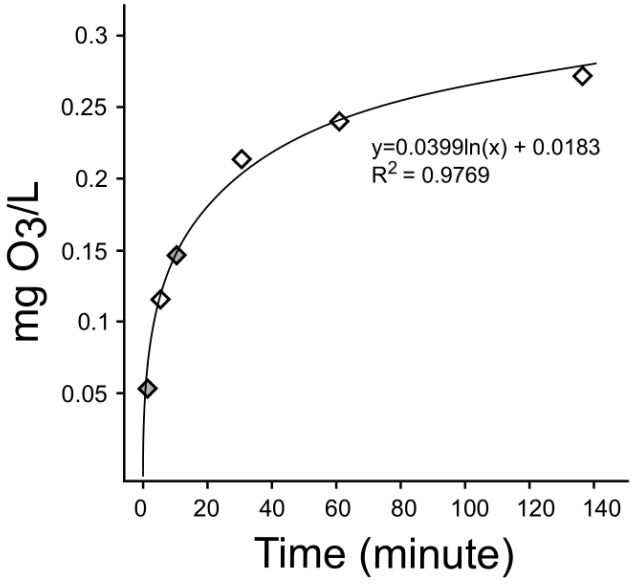

Figure S1. Ozone concentrations (mg O_3/L) in the ozonation vessel (250 mL dechlorinated carbon filtered water at ±25 °C) sampled at 1, 5, 10, 30, 60, and 135 minutes after initiation of the ozonation process. The filled diamonds represent the low (1 minute) and high (10 minutes) ozonation dosages which were applied to the pharmaceutical solutions.

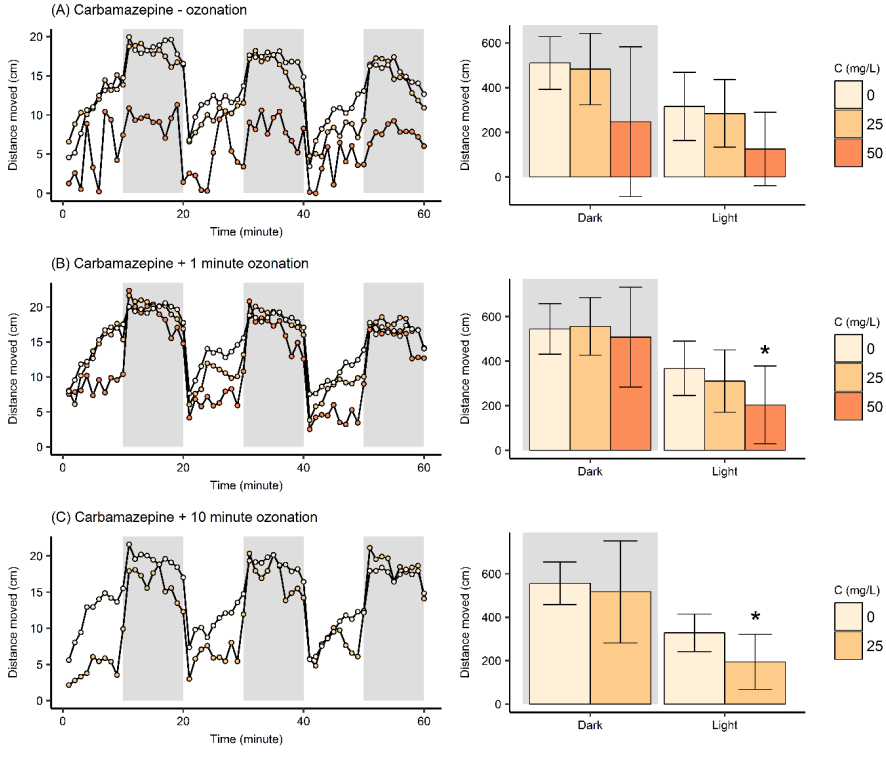

Figure S2. Locomotor activity (distance moved over time) in zebrafish exposed to carbamazepine ozonated 0 minutes (A), 1 minute (B) and 10 minutes (C). The left panel show mean total distance moved per minute and the right panel shows mean total distance moved during dark and light conditions. Larvae displayed hypoactivity during light conditions after exposure to 50 mg/L carbamazepine ozonated during 1 minute (B; ANOVA $p = 0.05709$, Dunnett's $p<0.05$) and 25 mg/L during 10 minutes (C; ANOVA $p = 0.01003$, Dunnett's $p<0.05$).

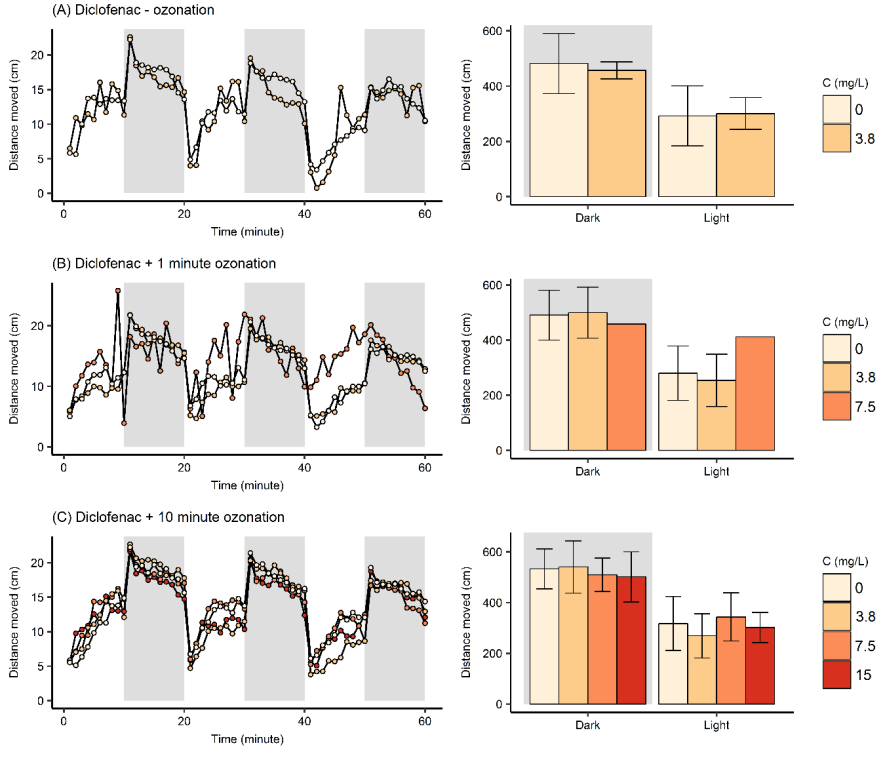

Figure S3. Locomotor activity (distance moved over time) in zebrafish exposed to diclofenac ozonated 0 minutes (A), 1 minute (B) and 10 minutes (C). The right panel show mean total distance moved per minute and the right panel shows mean total distance moved during dark and light conditions.

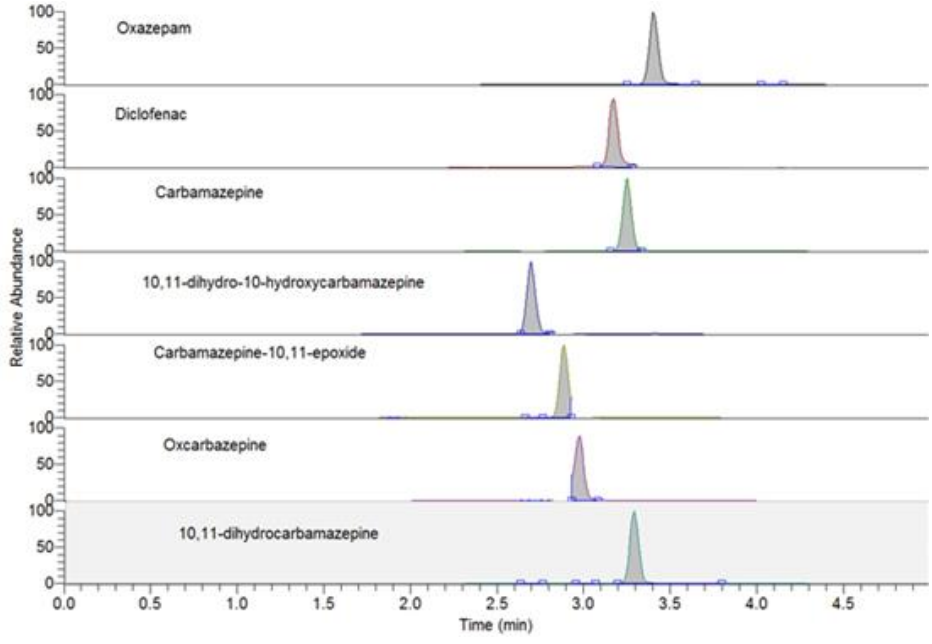

Figure S4. Chromatograms for the target compounds.

Recoveries. The recovery of the target compounds was studied by spiking control ozonated water with a mixture of the target compounds. The average recovery of target compounds was 100±5% (recovery was tested for one concentration levels, duplicates).

The average limit of quantification for the target compounds were: Oxazepam 0.003 mg/L, diclofenac 0.004 mg/L, carbamazepine 0.003 mg/L, 10,11-dihydro-10-hydroxycarbamazepine 0.008 mg/L, carbamazepine-10,11-epoxide 0.004 mg/L, oxcarbazepine 0.003 mg/L and 10,11-dihydrocarbamazepine 0.004 mg/L.

Figure S5. Representative images of observed embryo malformations.

III

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

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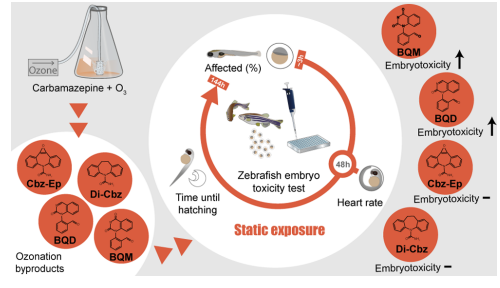
ABSTRACT: Carbamazepine (CBZ) is an anticonvulsant medication with highly persistent properties in the aquatic environment, where it has the potential to affect nontarget biota. Because CBZ and many other pharmaceuticals are not readily removed in conventional sewage treatment plants (STP), additional 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-(1*H*,3*H*)-quinazoline-2-one (BQM), and 1-(2-benzaldehyde)-(1*H*,3*H*)-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 ozonated 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.[1] Carbamazepine (CBZ) is an anticonvulsant pharmaceutical mainly prescribed to patients suffering from epilepsy and also used to medicate symptoms of schizophrenia and bipolar disorder.[2] 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.[3,4] The mode of action for CBZ is through sodium channel blocking by the therapeutically active carbamazepine 10,11-epoxide (CBZ-EP) metabolite,[5,6] reducing synaptic activity in the central nervous system.[7] About 13% of the ingested CBZ dose is excreted in unmetabolized form, mainly fecal excretion.[8]

The overall removal efficiency of CBZ in conventional STPs is only about 2%.[9] CBZ is consequently detected in surface water environments in the ng to μg L^{-1} concentration range.[10−12] CBZ is furthermore not prone to photodegradation.[13] 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.[9] Bioaccumulation of CBZ has been observed in, for example, bivalves[14] and fish.[15] Adverse effects resulting from CBZ exposure at concentrations proximal to environmental levels have been demonstrated in aquatic invertebrates.[16] Fish are however considerably less sensitive to CBZ, with acute and chronic effect concentrations reported in the 10−100 mg L^{-1} range.[16−18] 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.[19]

Novel technologies with the aim to improve STP removal efficiencies of pharmaceuticals and other micropollutants are being investigated.[20] Studies on ozonation as a tertiary STP effluent treatment technology have shown that CBZ can be removed by more than 90%.[21−23] However, ozonation of CBZ

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Table 1. Retention Time (RT), Molecular Mass (M), Extinction Coefficient (ε), Wavelength (λ), Absorption (A), Concentration (C), and Amount (m) of CBZ and Isolated OBPs

compound	RT (min)	M (g mol^{-1})	ε	λ (nm)	A	C (mmol L^{-1})	m (mg)
CBZ	10.7	236	11900[a]	284	594998	0.1059	1.50
BQM	8.8	250	35000[b]	206	1876920	0.2272	0.57
BQD	9.4	266	28000[b]	219	394158	0.0596	0.16

[a]Measured at 248 nm. [b]McDowell et al. (2005).[28]

can cause increased toxicity as observed in zebrafish (*Danio rerio*) embryos,[17] and cell-based in vitro assays.[24,25] 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-hydro-(1*H*,3*H*)-quinazoline-2-one (BQM) and 1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione (BQD), have been identified previously.[26−28] Besides, 10,11-Dihydrocarbamazepine (DI-CBZ) and the therapeutically active CBZ metabolite CBZ-EP have been quantified after ozonation of CBZ.[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). An internal standard of CBZ (D_{10}) was acquired from Sigma-Aldrich (Sweden). Ethyl 3-amino-benzoate methanesulfonate salt (MS-222) was purchased from Sigma-Aldrich (Sweden). Ultrapure water was produced by a Milli-Q Advantage ultrapure water purification system and filtered through a 0.22 μm Millipak express membrane and an LC-Pak polishing unit (Merk 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 mS cm^{-1}, alkalinity: 8 °dH, dissolved O_2: 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 O_3) using a lab-scale ozone generator (described in detail in the report by Pohl et al.[17]). Dissolved O_3 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 1.

$$A = \varepsilon \times C \times l \quad (1)$$

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

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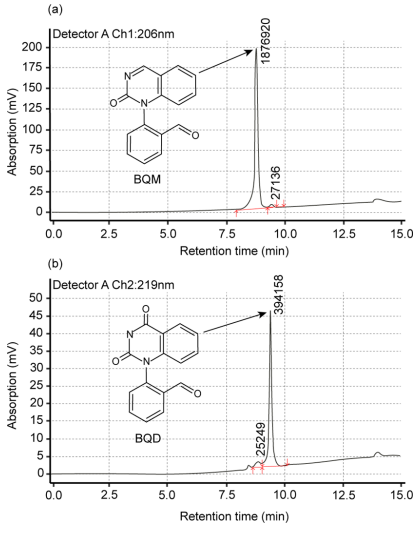

Figure 1. High performance liquid chromatography chromatogram of (a) peak 1 (BQM, 206 nm) and (b) peak 2 (BQD, 219 nm) after isolation.

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 °dH, dissolved O_2: 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.[17] 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 time-points. 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

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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.[29,30] 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.[32] 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

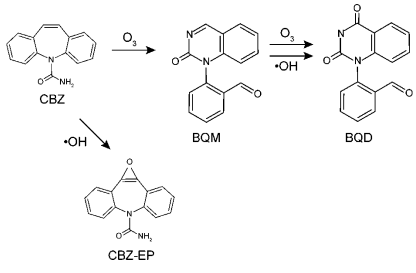

Figure 2. Proposed ozone-mediated degradation of CBZ, based on Figure 2 in the publication by Hübner et al. (2014).[27]

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 treatment, 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.[35,36] 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 diffusor 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,

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Table 2. Measured CBZ and OBP Concentrations (mg L^{-1}, Mean ± Standard Deviation, NA = Not Analyzed, LOQ = Limit of Quantification)

		Pohl et al.[17] (17 mg L^{-1} CBZ ozonated 10 min)	present study (17.5 mg L^{-1} CBZ ozonated 10 min)	single compound and mixture exposure solutions			
	abbreviation	1×	1×	0.5×	1×	2×	LOQ (mg L^{-1})
carbamazepine	CBZ	<LOQ	3.1	<LOQ	<LOQ	<LOQ	0.0025
carbamazepine 10,11-epoxide	CBZ-EP	0.5	0.5	0.8	1.5	2.6	0.0024
10,11-Dihydrocarbamazepine	DI-CBZ	0.2	0.02	0.1	0.2	0.4	0.0024
1-(2-benzaldehyde)-4-hydro-(1*H*,3*H*)-quinazoline-2-one	BQM	NA	2.2	0.8	1.8	3.5	0.0026
1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione	BQD	NA	5.5	1.7	1.9	6	0.0019
mixture	CBZ-EP			0.7	1.9	4.3	0.0024
	DI-CBZ			0.1	0.2	0.5	0.0024
	BQM			0.8	1.6	3.7	0.0026
	BQD			1.5	2.6	7.7	0.0019

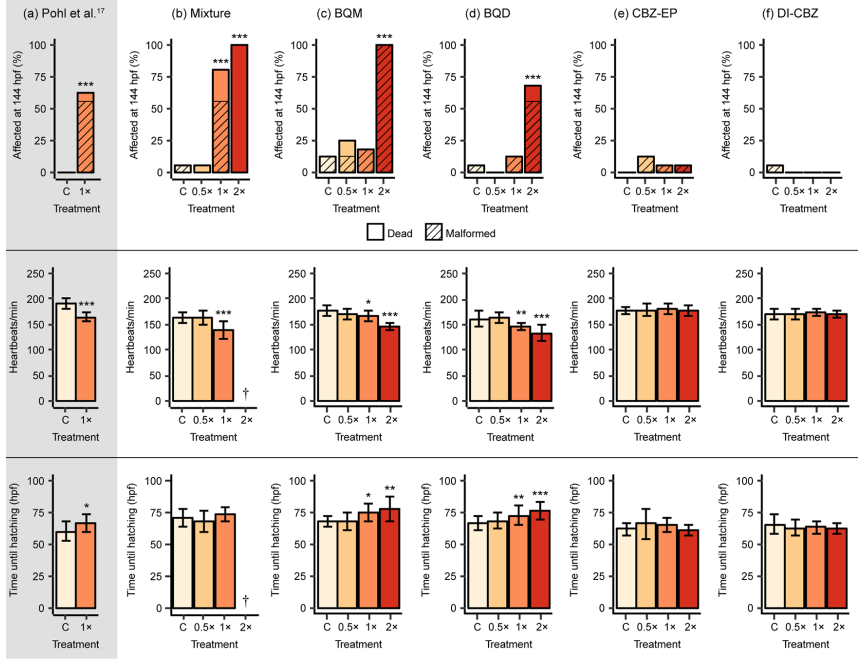

Figure 3. Embryotoxicity responses, that is, proportion of affected (dead and/or malformed) embryo–larvae (%), heart rate (beats min^{-1}, mean ± sd), and time until hatching (hpf, mean ± sd) in zebrafish exposed to (a) ozonated CBZ (adapted from Pohl et al.[17]), (b) mixture, (c) BQM, (d) BQD, (e) CBZ-EP, and (f) DI-CBZ. The treatment groups correspond to approximately half (0.5×), equal (1×), and double (2×) the concentrations measured in ozonated CBZ. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. †: all embryos were dead at the time of measurement.

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×

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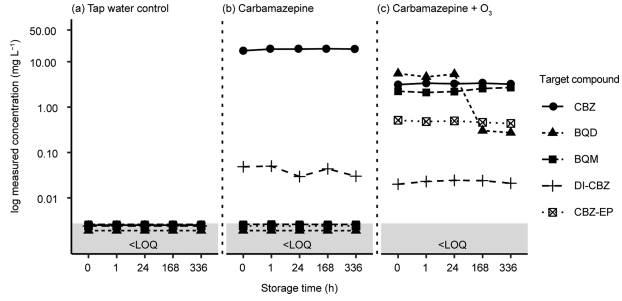

Figure 4. Measured concentrations (mg L^{-1}) of target compounds in (a) tap water control, (b) carbamazepine, and (c) ozonated CBZ in tap water stored for 0, 1, 24, 168, and 336 h. The gray area signifies target compound LOQ ranges (maximum average LOQ: 0.0025 mg L^{-1}).

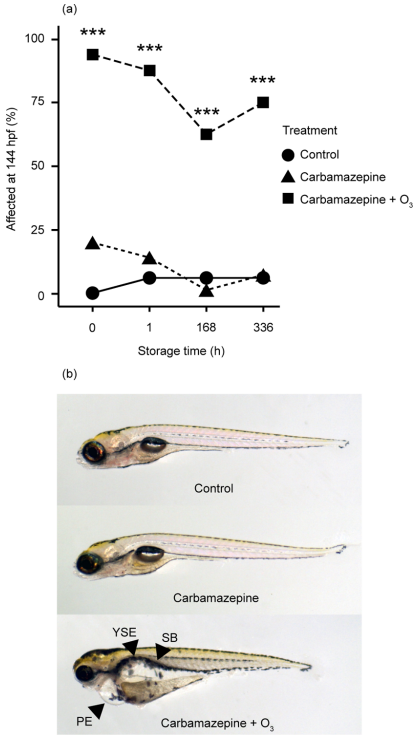

Figure 5. (a) Proportion of affected (dead and malformed) zebrafish embryo–larvae at 144 hpf after exposure to tap water control, 17.5 mg carbamazepine L^{-1}, and 17.5 mg carbamazepine L^{-1} + O_3 stored for 0, 1, 168, and 336 h in the dark at 22 °C. Significant differences as compared to controls 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 + O_3 (PE, SB, YSE, and general growth retardation) at 144 hpf.

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.[17] 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.[17]) 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 (Table 2). 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.[17]). The two other tested putative 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.[25]

3.4. Stability and Embryotoxicity of Pre- and Postozonated CBZ. Because CBZ has recalcitrant properties in the aquatic environment,[9] 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

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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.[27] 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 5a). 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.[17] 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.[17]

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.[27] 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 O_3, 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/MS TSQ Quantiva (PDF)

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

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

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Table S1. MS/MS parameters for a triple-stage quadrupole MS/MS TSQ Quantiva.

Compound	**Abbreviation**	**Retention Time (min)**	**Polarity**	**Precursor (m/z)**	**Product (m/z)**	**Type**	**Collision Energy (V)**
Carbamazepine	CBZ	5.4	Positive	237.102	194.096	Quan	20
					192.081	Qual	24
Carbamazepine 10, 11-epoxide	CBZ-EP	4.7	Positive	253	180.058	Quan	28.66
					210.091	Qual	20
10,11-dihydrocarbamazepine	DI-CBZ	5	Positive	239	194.04	Quan	23.3
					180.058	Qual	39.53
1-(2-benzaldehyde)-4-hydro-(1*H*,3*H*)-quinazoline-2-one	BQM	5.2	Positive	251.082	180.081	Quan	30
					208.076	Qual	30
1-(2-benzaldehyde)-(1*H*,3*H*)-quinazoline-2,4-dione	BQD	6	Positive	267.077	167.073	Quan	30
					196.076	Qual	30
Carbamazepine-D10		5.3	Positive	247.212	204.169	Quan	22.59

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Doctoral Thesis No. 2020:5

Pharmaceuticals pollute the aquatic environment, causing adverse effects in fish and other organisms. Implementing ozonation as a sewage treatment is a potential approach to improve pharmaceutical removal. This thesis evaluated ozonation of a sewage effluent and selected pharmaceuticals in zebrafish (*Danio rerio*). Ozone treatment gave rise to diverging toxicity outcomes, both beneficial and adverse. Consequently, the formation and toxicities of ozonation by-products were studied. Ozonation as a sewage treatment method needs to be carefully monitored regarding toxicity.

Johannes Pohl received his doctoral education at the Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences. His bachelor's and master's degrees were obtained at Uppsala University.

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