Ozonation as sewage effluent treatment

Toxicological effects in adult and embryonic zebrafish (*Danio rerio*)

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Abstract

Pharmaceutical residues are not efficiently removed by current sewage treatment plant (STP) technologies. This allows for the release of pharmaceutical residues into the aquatic environment. Pharmaceuticals and their metabolites are problematic because they are designed to affect biological processes in non-target aquatic species. It is therefore of interest to limit the release of pharmaceutical residues in order to protect the surface water environment. One potential method for the improvement of sewage effluent treatment is ozonation, a technology capable of eliminating pharmaceutical residues.

This licentiate thesis aimed to evaluate the biological effects in adult and embryonic zebrafish (*Danio rerio*) exposed to ozonated effluent at a Swedish municipal STP outfitted with a full scale effluent ozonation step. Reproduction-related endpoints (e.g. fecundity and gonad maturation), behaviour and hepatic gene expression (e.g. vitellogenin) were sampled in fish exposed for 21 days. Responses in fish exposed to ozonated STP effluent diverged from fish exposed to the normal, non-ozonated STP effluent and tap water control. Firstly, they produced twice the amount of fertilized eggs and had a higher degree of gonadal maturation. Secondly, male hepatic vitellogenin gene expression was induced, indicating estrogenicity due to ozonation. Thirdly, fish exposed to the ozonated effluent effluent exhibited a stress-like behaviour.

While ozonation has proven to be very efficient in reducing pharmaceutical parent compound concentrations in STP effluents (on average 77% at Knivsta STP), much remains unclear regarding potentially toxic ozonated by-product (OBP) formation. Therefore, the second part of the thesis explored how zebrafish embryotoxicity of three pharmaceuticals of environmental relevance would be modulated by ozonation in a bench-scale ozonation study. Carbamazepine embryotoxicity increased following ozonation, possibly explained by formation of the OBP carbamazepine-epoxide. Furthermore, ozonation of oxazepam seemingly potentiated its anxiolytic mode of action in exposed zebrafish larvae. Diclofenac embryotoxicity was however completely abolished by ozonation.

The results presented in this thesis highlight the importance of new chemical and toxicological knowledge regarding the formation of OBPs in post-ozonated effluents.

Keywords: Zebrafish reproduction, zebrafish behavior, zebrafish embryotoxicity, Municipal sewage treatment, ozonation-by products, pharmaceutical residues

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Ozonering som avloppsreningsmetod. Toxiska effekter i vuxen och embryonal sebrafisk (*Danio rerio*)

Sammanfattning

I nuläget riskerar läkemedelsrester från att läcka ut i vattenmiljön då dessa inte avskiljs effektivt i våra reningsverk. Detta är problematiskt eftersom läkemedel och deras metaboliter har förmågan att kunna påverka biologiska processer hos vattenlevande organismer. Därför är det av intresse att begränsa utsläppen av läkemedelsrester för att kunna skydda dricksvattenkällor och akvatiska ekosystem. En möjlig lösning för att förbättra avloppsvattenrening vid reningsverken är ozonering av den utgående effluenten.

Denna licentiatavhandling syftade till att utvärdera de biologiska effekterna hos vuxna och embryonala sebrafiskar (*Danio rerio*) exponerade för ozonerad effluent och utvalda läkemedel. Försöket med ozonerat avloppsvatten skedde på ett kommunalt reningsverk i Knivsta, Sverige, där ett fullskaligt ozoneringssteg hade installerats. Fortplantningsförmågan (t.ex. antal befruktade ägg och gonadmognad), beteende och genuttryck (t.ex. vitellogenin) mättes i fisken som var exponerad under 21 dagar. Fiskarna som var exponerade för ozonerad effluent skilde sig åt från de fiskar som var exponerade för den vanliga effluenten samt dricksvattenkontrollen. De producerade dubbelt så många befruktade ägg och hade en högre grad av gonadmognad. Hanfiskarnas vitellogenin-uttryck i levern var upreglerat, vilket indikerar ökad estrogenicitet i den ozonerade effluenten. De uppvisade även ett förmodat stress-relaterat simbeteende.

Trots att ozonering har visat sig vara ett mycket effektivt sätt att minska concentrationen av läkemedelssubstanser i effluenten (i genomsnitt 77 % i Knivsta reningsverk) kvarstod många frågetecken kring hur skadliga eventuella ozoneringsbiprodukter är. Därför ägnades den andra halvan av avhandlingen åt undersökningar av zebrafiskembryotoxicitet kopplad till tre ozonering av tre läkemedel med miljömässig relevans (karbamazepin, diklofenak och oxazepam). Karbamazepinets embryotoxicitet ökade efter ozonering, vilket skulle kunna kopplas ihop med att OBP formationen karbamazepin-epoxid bildades. Ozoneringen av oxazepam ledde till att dess ångestdämpande effekt förstärktes. Diklofenaks embryotoxicitet eliminerades helt efter ozonering. Resultaten som presenteras i denna licentiatavhandling understryker behovet av ny kemisk och toxikologisk kunskap kring de ozoneringsbiprodukter som kan uppstå i ozonerade effluenter.

Nyckelord: Sebrafisk, avloppsrening, läkemedelsrester, ozoneringsbiprodukter

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Dedication

To Johanna

In nature nothing exists alone.
Rachel Carson

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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. 2018. Embryotoxicity of ozonated diclofenac, carbamazepine, and oxazepam in zebrafish (*Danio rerio*). Manuscript.

Paper I is reproduced with the permission of the publisher.

* 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

II JP had the main responsibility of designing and executing the experimental work of the manuscript. Oksana Golovko also contributed experimentally by performing the chemical analysis part of the manuscript. JP had the main responsibility for compiling literature and writing the manuscript with support from all co-authors.

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1 Introduction

1.1 Pharmaceuticals in the aquatic environment

The surface water environment and its diverse aquatic and semi-aquatic life is – undeniably – of countless value. Unfortunately, it is also the destination for organic pollutants originating from human activity (Daughton and Ternes, 1999). Pharmaceuticals are significant contributors to this pollutant cocktail. One major route of pharmaceuticals into the surface water environment is through discharges from sewage treatment plants (STPs). Given the propensity of the growing global community to treat disease in human patients and livestock, the use of pharmaceuticals is set to increase. Two considerable areas of concern are emerging from this fact; accelerated antibiotic resistance in pathogenic microorganisms and adverse effects in aquatic organisms (Larsson, 2014).

Raw sewage influent typically consists of domestic wastewater including urine and faeces, paper products, and detergents. Pharmaceutical and personal care product (PPCP) residues also end up in domestic wastewater. Pharmaceuticals, in particular, are present as either therapeutically active, or as metabolized and excreted by the patient in the urine or faeces. Conventional STPs have been quite successful in reducing the chemical oxygen demand (COD) and biochemical oxygen demand (BOD) of the incoming sewage water. COD and BOD are a measure of the dissolved oxygen required for chemical and microorganism facilitated ammonia oxidation in raw sewage. If COD and BOD are not adequately reduced before release into the receiving water body, diminished oxygen levels could be the outcome. This can ultimately lead to direct detrimental effects to the flora and fauna. While this outcome is important

to prevent, concentrations of pharmaceuticals and their metabolites remain largely unaffected by current practices.

Pharmaceutical production facilities are considered hot spots of pharmaceutical pollution. An STP located in a heavily industrialized part of Hyderabad, India, produced an effluent containing as much as 44 kg ciprofloxacin per day, as compared to the national prescribed use in Sweden of 9 kg (Larsson et al., 2007). Evidence of widespread presence of pharmaceuticals in surface water, mainly due to inadequate sewage treatment, has been reported in several publications (e.g. aus der Beek et al., 2016; Hughes et al., 2013). Ternes (1998) studied how a number of selected pharmaceuticals passed through a conventionally equipped German STP (Frankfurt am Main) over the course of 6 days. It was found that while two common pharmaceuticals, ibuprofen and acetylsalicylic acid, were efficiently removed (81 and 90 %, respectively), the antiepileptic drug carbamazepine was not significantly reduced (7%). Some of the selected compounds, including diclofenac and bezafibrate, were detected far downstream (up to 77 kilometers) from the STP. The pooled environmental risk of pharmaceuticals serious is considered to be serious on a continental scale (Malaj et al., 2014). As a consequence, regulations are implemented to reduce the load of pharmaceutical contamination in the aquatic environment; for example, diclofenac, along with 17-beta-estradiol, and 17-alpha-ethinylestradiol, have been added to the 2013/39/EU directive watch list of priority substances (European Union, 2015).

1.2 Effects of sewage effluents on fish

Research indicating detrimental effects in wild freshwater species exposed to raw wastewater effluents has illustrated the need to improve the chemical removal efficiency. One of the earliest biomarkers put in use by scientists to illuminate adverse effects in response to pollutants in wastewater effluents was the abnormal increase of plasma vitellogenin in male fish. Vitellogenin, a yolk-protein precursor molecule expressed in the fish liver as a response of estrogen, is normally produced in the female fish prior to ovulation. After noticing how fish in ponds of STP effluent had a high incidence of intersex, Purdom et al., (1994) reported that caged male rainbow trout (*Oncorhynchus mykiss*) in UK effluent influenced waters produced an elevated expression of vitellogenin. Routledge et al. (1998) further investigated the vitellogenin induction in male rainbow trout and adult roach (*Rutilus rutilus*) exposed in the lab to concentrations of the steroidal estrogens relevant to measured UK STP effluents, and corroborated earlier claims. Roach exposed to UK STP effluents from fertilization up to 300 days of age exhibited kidney damage, genotoxic and

immunotoxic response on top of overt sexual endocrine disruption (Liney et al., 2005). These indications are however are not confirmed by other studies on fish exposed to conventional STP effluents that fail to detect sexual endocrine disruption. Wild gudgeon (*Gobio gobio*) from UK rivers, sampled for the same physiological and vitellogenin biomarkers as Purdom et al. (1994), did not show similar effects (van Aerle et al., 2001). In the US, Nichols et al. (1999) used fathead minnow (*Pimephales promelas*) to measure female ovarian status and male gonadal score and plasma vitellogenin concentration. The authors compared these physiological and endocrine biomarkers in wild fish from a reference site to fish caged at several different STP effluent enriched waterbodies. Their results were not able to conclusively establish that STP effluent in itself adversely affected these biomarkers.

Another studied biomarker involved in the maturation of eggs, gonadal CR/20beta-HSD gene induction, has been observed in juvenile *O. mykiss* caged downstream of a Swedish STP (Albertsson et al., 2010). Fish exposed to single estrogenic (10 ng ethinylestradiol/L) compounds did not exhibit this response in the same study (Albertsson et al., 2010). Likewise, reduced egg spawning in zebrafish (*Danio rerio*) exposed to a 50% diluted municipal STP effluent, but not 10 ng/L ethinylestradiol, was reported by Lister et al., (2009). It is thus probable that the combined mixture of pharmaceuticals in an STP effluent can elicit a stronger adverse biological response than individual compounds. Brian et al., (2007) illustrated this very effect in egg spawning fathead minnows exposed to a mixture of estrogenic chemicals. (Björkblom et al., 2009), Ma et al., (2005) and Thorpe et al., (2009) have also reported diminished reproduction success in fish exposed to STP effluents. The indications of adverse effects in fish, including effects that have the potential to disturb whole populations, is alarming. One method of wastewater treatment that has the potential to curb pharmaceutical pollution in the surface water environment is ozonation of STP effluents.

1.3 Ozonation as an additional sewage treatment step

Strategies to address the pharmaceutical contamination of aquatic environments can be designated upstream and downstream actions. Upstream actions (input prevention) aims to prevent pharmaceutical pollution in the first place by e.g. improved legislation and regulation (Ågerstrand et al., 2015); downstream actions deal with already existent pharmaceutical pollution (end-of-pipe solutions). The scope of this licentiate thesis cover evaluation of ozonation as an end-of-pipe solution.

A shift towards investments in additional treatment steps to mitigate pollution has been taking place during the last couple of decades (Eggen et al., 2014). One such technology is ozonation of the STP effluent. Ozone has the capacity to remove pharmaceuticals from STP effluents. (Lavén et al., 2009) analyzed the ozonated wastewater effluent from a Swedish STP. The same effluent was subsequently characterized by a number of biological studies on fish (Albertsson et al., 2010; Cuklev et al., 2012; Gunnarsson et al., 2009; Lundström et al., 2010; Samuelsson et al., 2011). Ozonation proved to be a very efficient (>90%) removal method of 15 analyzed pharmaceuticals including carbamazepine, propranolol and other persistent compounds (Lavén et al., 2009). Synthetic estrogens has been shown to be effectively removed by 95-99% in a study using ozonation as an additional STP effluent treatment step (Sun et al., 2017).

Undoubtedly, ozone could be ascribed to be an effective removal agent for certain known environmental contaminants. However, it remains important to explore how the ozonation treatment modulates the biological effect in aquatic organisms exposed to the STP effluent due to the suspicion of the effects of ozonation by-products (OBPs).

1.4 Ozonation by-products

It is important to bear in mind at this point that apparent removal of a chemical based on analytical chemistry detection methods does not necessarily mean complete mineralization. The fate of the ozonated pharmaceuticals, and other organic molecules present in the STP effluent, is potentially dependent on a multitude of factors including ozone contact time and concentration. Partially degraded OBPs are formed during the ozonation procedure. The production of certain OBPs has been reported and discussed in the scientific literature (Escher and Fenner, 2011, von Gunten, 2003, Lee and von Gunten, 2016). Yan et al. (2014) detected increasing concentrations of aldehydes following increasing ozone dosing in unfiltered STP effluents. The suspected carcinogenic compounds N-Nitrosodimethylamine (NDMA) and bromate was detected in an ozonated STP effluent in a study by Zimmermann et al. (2011), but the concentrations of NDMA declined after the post-ozonation filter. The bromate concentrations were however not affected by the filter (Zimmermann et al., 2011).

We are likely to be walking a fine line in order to achieve removal of pharmaceutical residues while avoiding increased toxicity, due to OBPs, in ozonated STP effluents.

2 Objectives

This licentiate thesis aimed to explore the ecotoxicological risks and/or benefits of ozonation treatment of STP effluents, with a focus on pharmaceutical toxicity in zebrafish. The thesis work was divided into two parts. Firstly, a Swedish STP (Knivsta municipal STP) with a full-scale ozonation step was evaluated for biological effects in reproduction and behavior of zebrafish (Paper I). Questions regarding the technical and financial feasibility of whole-effluent ozonation strategies lie outside the scope of this thesis. The second part of the thesis focused on how the embryotoxicity of selected individual pharmaceuticals detected in the Knivsta STP effluent was modulated by ozonation on a lab bench scale (Paper II). The thesis encompassed the following research questions:

- Is ozonation of STP effluent beneficial or not for the biological status (reproduction, behavioral symptoms of stress etc.) in exposed zebrafish?

- How may ozonation modulate embryotoxicity of the three common pharmaceutical contaminants carbamazepine, diclofenac, and oxazepam?

3 Materials & Methods

This chapter features a summarized overview of the methods and techniques further elaborated upon in Papers I and II.

3.1 Experimental settings

3.1.1 Knivsta municipal sewage treatment plant (Paper I)

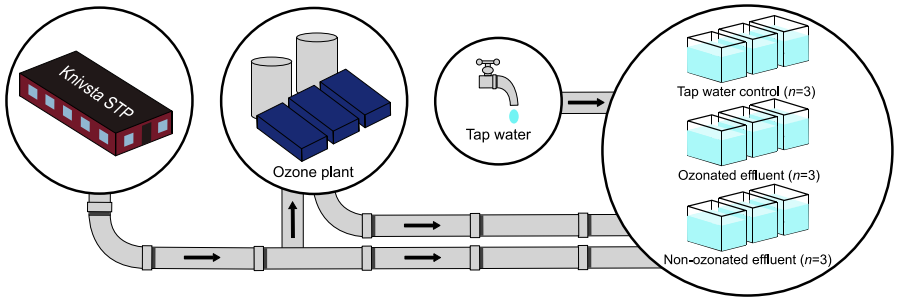

Figure 1. The experimental setting in Paper I.

Knivsta is a Swedish municipality located between Stockholm and Uppsala. The Knivsta municipal STP has a treatment capacity of 13 000 population equivalents and is situated slightly south of the city center along the Knivsta River. Knivsta STP has five major treatment steps: pretreatment, primary sedimentation with chemical precipitation, biological treatment, secondary sedimentation, and post-chemical precipitation. After treatment, the effluent is released into constructed wetlands from where it is released in Knivsta River. The Knivsta River flows southward into Lake Mälaren and subsequently the Baltic Sea. For the occasion of the study presented in Paper I, a full-scale ozonation plant was constructed and operated in the proximity of the effluent

outlet. The plant was calibrated to continuously inject 7 g O_3 per m^3 treated effluent. A mobile laboratory (isolated 10 ft ISO-container) containing aquariums (*n*=3 per treatment) for the fish studies was installed next to the ozonation plant. Water from the effluent, ozonated effluent, and the municipal tap water system was continuously supplied to the mobile laboratory. Adult zebrafish were exposed on-site over 21 days. A schematic representation of the experimental design in Paper 1 is shown in Figure 1.

3.1.2 Bench ozonation set-up (Paper II)

Chemicals

All chemicals were sourced from major resellers and of analytical grade. More information on the chemicals used for each study is found in the presented Papers I and II.

Experimental ozone generator set-up

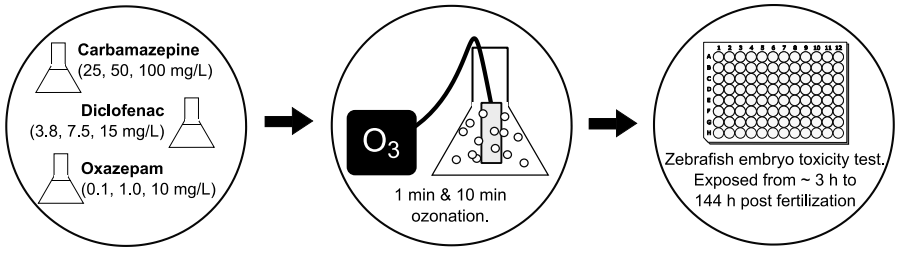

Figure 2. Schematic representation of the experimental setting in Paper II.

The experimental ozonation reactor used for Paper II was set up on site at the aquatic laboratories at VHC, SLU. An ozone generator (AB Aqua Medic GMBH, Bissendorf, Germany) supplying 100 mg O_3 per hour was used to ozonate test solutions (v=250 mL, ±25 °C) by way of diffusion stone in Erlenmeyer flasks. The set up was localized to a fume hood in order to dissipate residual ozone gas. A schematic representation of the ozonation experimental set-up is shown in Figure 2.

A preliminary test of peak ozone was performed to evaluate ozonation efficiency (Figure 3). Peak ozone concentrations over time were measured using the LCK310 Ozone cuvette test (0.05-2 mg/L O_3 measurement range) in a DR 3900™ spectrophotometer (Hach, Loveland, Colorado, United States). From the preliminary test, two ozone dosages were selected for subsequent treatment of

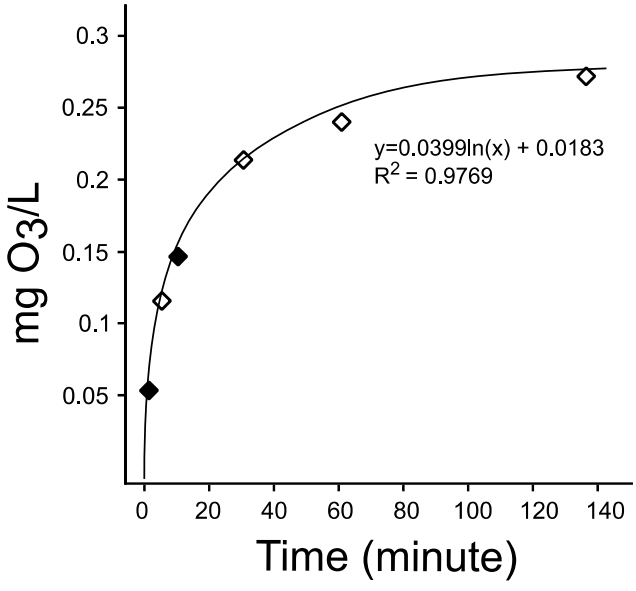

Figure 3. Ozone concentrations (mg O_3/L) in the ozonation vessel (250 mL dechlorinated carbon filtered water at ±25 °C) sampled at 1, 5, 10, 30, 60, and 135 min after initiation of the ozonation process. The filled diamonds represent the low (1 min) and high (10 min) ozonation dosages which were applied to the pharmaceutical solutions.

the tested pharmaceuticals; 1 min ozonation (peak ozone concentration of 0.053 mg ozone/L), and 10 min ozonation (peak ozone concentration of 0.147 mg ozone/L). The rationale for choosing these two dosages were mainly due to practical feasibility. In order to complete ozonation and initiate the embryotoxicity experiments (see section 3.2.2) during the same morning, additional or longer ozonation dosages would not have been possible.

3.2 Experimental techniques

3.2.1 Adult zebrafish (paper I)

The zebrafish is a fish species in the freshwater cyprinid family, native to the Indian subcontinent. It is a small fish with an adult size of about 3-4 centimeters, living in streams and shallow ponds. Reproduction is performed externally. During courtship female expels its eggs, and the male releases sperm on the eggs as they drop to the bottom. The zebrafish is considered a popular model species in a wide range of scientific fields, from the behavioral and medical sciences to

environmental toxicology. This owes to the fact that zebrafish reproduce easily under controlled laboratory settings. Furthermore, the eggs are completely transparent which allows inspection of embryo morphology and bodily functions (i.e. heart rate) by stereo microscope. The embryo normally develops to sexual maturity in a matter of months, enabling multigenerational studies. The whole genome has been sequenced and extensively annotated. The complex zebrafish behavioral repertoire has also been studied and is suggested to be robust enough to support claims on neurobehavioral effects of medicines aimed for human consumption. All in all, these characteristics promoted the use of zebrafish in the studies presented in this thesis. All experimentation on adult fish were approved beforehand by the Animal Ethics Committee in Gothenburg (DNR79-2014).

Reproduction assay

The zebrafish reproduction is stimulated by turning on the lights in the morning. Each aquarium population, with five putative males and five putative females, is considered a replicate and the observation is the total amount of fertilized eggs spawned per breeding trial (0.5 h duration). The aquarium population is herded into submerged stainless steel cages for the duration of the breeding trial. Any spawned eggs fall to the bottom of the aquarium and siphoned up and is counted and assessed for fertilization success.

Behavior assay

The zebrafish behavior was tested in a novel tank test, where individual fish are transferred from their home tank into separate small tanks. This action should produce an anxiety-like response in fish quantified by measuring swimming activity over time. A stressed zebrafish will freeze when introduced to a novel environment, before starting to investigate its surroundings. This metric for anxiety-like response is a simplified version of more sophisticated methods, including time spent bottom-dwelling and response to visual or olfactory distress cues (Kalueff et al., 2013).

Histology

Investigation of the female gonad for proportions of different maturity stages of oocytes was performed. The male gonad was also investigated for signs of effects. The gonads were excised from the fish after being euthanized and prepared for histological sectioning. Prepared sections were analysed under stereo microscope.

Quantitative real-time RT-PCR

Quantitative gene expression analysis was measured by a Rotor-Gene 3000 instrument (Corbett Research, Australia) using 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. The zebrafish hepatic mRNA was homogenized by a Precellys Evolution (Bertin instruments, France) system equipped 190 with liquid nitrogen cooling. The RNA integrity and concentration were measured in a 2100 Bioanalyzer system (Agilent Technologies, USA) to ensure sample template quality. Relative mRNA expression was calculated by the $E^{-\Delta\Delta ct}$ method described by (Livak and Schmittgen, 2001).

3.2.2 Zebrafish embryos (Papers I and II)

The zebrafish embryo is suitable for developmental toxicity studies. Due to the rapid nature of zebrafish reproduction, a large number of eggs can be collected daily from a breeding stock of adult zebrafish. After fertilization, the eggs are separated and exposed individually in microwell plates. In this way, each embryo can be treated as one experimental replicate since it is contained in its own exposure medium for the duration of the experiment. No interaction between embryos which may alternate biological responses is taking place. This enables a rapid high-throughput test system for sublethal and lethal effects of different environmental matrices (e.g. wastewater effluent samples in Paper I) or selected concentrations of chemicals (e.g. pharmaceuticals in Paper II).

The zebrafish embryo test represents a robust whole organism ecotoxicity test for detecting effects on lethal and sublethal endpoints (Hill et al., 2005). This methodology has been used in previous STP effluent ozonation toxicity studies (Lundström et al., 2010; Wigh et al., 2015). Furthermore, we combined this assay with a larvae locomotor activity test for identifying behavioral effects. The larvae locomotor activity has been proposed as a rapid and valuable test for neurodevelopmental effects in fish larvae (Burgess and Granato, 2007).

Before commencing the ozonation tests, a concentration-response screening of each pharmaceutical was performed. Three suitable concentration intervals were identified and subsequently ozonated in order to evaluate how ozone would modulate embryotoxic response.

Lethal and morphological effects

Embryos were evaluated for lethal and observable morphological deformities under stereo microscope at 24, 48 and 144 hours post fertilization (hpf). Examples of morphological deformities due to exposure include pericardial edema, yolk sac edema, and scoliosis. The zebrafish embryotoxicity test performed as part of Paper I included only lethal and morphological effects as described here. Paper II applied heart rate and hatching time as additional endpoints.

Heart rate and hatching time

Heart rate was measured in the developing embryo at 48 hpf. Since the embryo is transparent, it is possible to visually count the number of heartbeats. In order to record the heart rate, a stop-watch was used to track the time while counting 30 heart beats. The heart rate (heartbeats per minute (bpm)) was then calculated by the formula

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

The hatching time was recorded by photographing (Canon EOS 500D) the whole microwell 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 determine the hour larvae hatched.

Larvae locmotor activity assay

The zebrafish larvae behavior in response to shifts in light intensity was measured using the Zebrabox tracking system and software (Viewpoint, France). 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.

3.3 Chemical analysis

Details of chemical analysis methodology are found in the respective Materials & Method sections of Paper I and II.

3.4 Statistics

The R software (R Core team, 2016), Rstudio interface (RStudio Team, 2016) and a collection of third-party packages (detailed in respective paper) were used for all statistical modeling, analysis, and plotting. Appropriate statistical methods and post-hoc tests were selected for parametric- and nonparametric data, elaborated upon in each respective paper.

4 Results & Discussion

4.1 Knivsta municipal STP effluent ozonation (paper I)

The field study at Knivsta STP (Paper I) was conducted during the winter months of 2015-2016. Its aim was to evaluate biologically the effects that ozonation in full scale would have on exposed fish. Furthermore, the removal success of pharmaceutical residues was investigated. Chemical analysis of STP effluent samples showed that 24 out of 105 target pharmaceuticals could be detected prior to ozonation at levels exceeding the limit of quantification (LOQ; Table 1). After ozonation, 11 pharmaceuticals remained, and the average removal efficiency was 77% (Table 1). Fluconazole (24%), oxazepam (42%) and irbesartan (44%) were least efficiently removed by the ozone treatment. Most other detected pharmaceutical were removed to a higher extent, with the examples of diclofenac (99%) and carbamazepine (97%). The results are largely comparable to other, similar studies of ozonation removal efficiency at STPs (e.g. Beijer et al., 2017). The chemical analysis did however not screen for the formation of any OBPs in the ozonated effluent (the OBP route of investigation was continued in Paper II).

4.1.1 Effects on zebrafish reproduction

Fish exposed to ozonated STP effluent produced approximately twice as many fertilized eggs than the conventional STP effluent and tap water control (Figure 4). In addition, histological evaluation of female gonads also revealed that the ozonated STP effluent treatment group had more vitellogenic stage oocytes (Figure 5), further indicating that reproduction functioned optimally.

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 pharmaceutical 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. Table adapted from Pohl et al., (2018).

Pharmaceutical	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	-	-

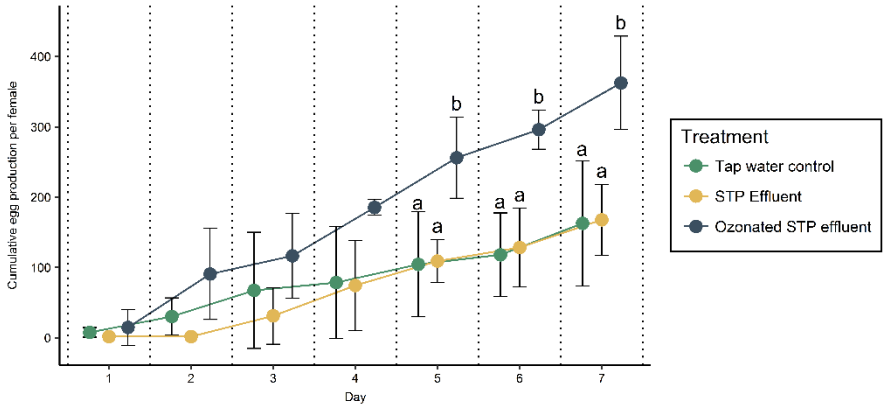

Figure 4. Cumulative number of fertilized eggs over the last 7 days of the 21 day exposure study at Knivsta STP (Paper I). Different letters indicates significant differences between groups ($p<0.05$). Figure adapted from Pohl et al., (2018).

A higher fecundity in zebrafish that we observed in Paper I is in line with previous research. Ward and DeGraeve, (1978) similarly sought to evaluate the biological effect arising in fathead minnows (*P. promelas*) when treating a STP effluent with ozone. They reported that no toxicity (either mortality, reproduction, or growth) was detected in *P. promelas* exposed to ozonated STP effluent. However, exposure to ozonated STP effluent seemed to induce egg production in fish to levels far above that of tap water control, similar to the

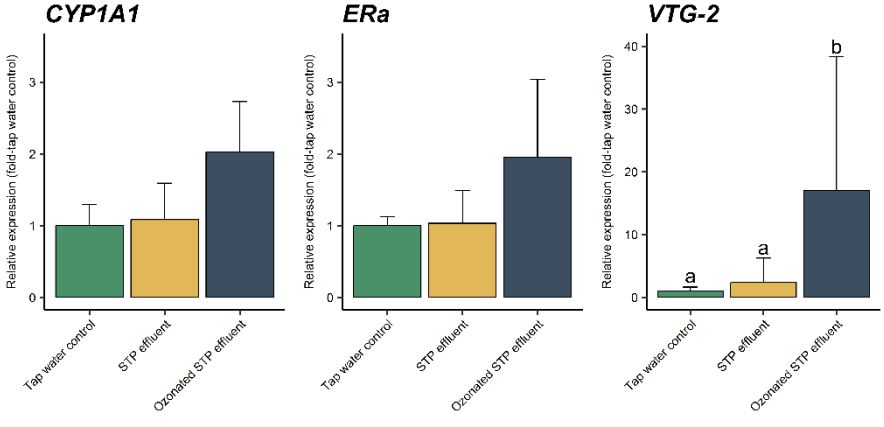

Figure 5. Gene expression (fold change – tap water control) of *ERα*, *CYP1A1* and *VTG-2* in male zebrafish liver tissue (n = 3, mean + sd). Different letters indicate significant differences (Tukey's HSD $p < 0.05$) in the expression of *VTG-2* (ANOVA $p = 0.0196$). The gene expression data was normalized against the household gene *ACBT* (the expression of which was not altered by any treatment). Figure adapted from Pohl et al., (2018).

outcome in Knivsta STP. One explanation for this response may be that the ozonated STP effluent represented an optimal breeding environment for the fish. Another explanation could be formation of OBPs with estrogenic potencies. Exposure to estrogenic compounds have been shown to promote reproduction in fish (Jobling et al., 2003; Rose et al., 2013). Male hepatic vitellogenin gene expression was induced in Paper I (Figure 6), which may in part explain the increased fecundity. This might indicate that the chemical composition of the effluent has become 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 17β-estradiol (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 normal effluent (Cuklev et al., 2012; Gunnarsson et al., 2009). The shifting outcomes of Paper I and similar studies emphasize the complexity of evaluating ozonation as an STP effluent treatment step. Yet it remains important to continuously evaluate the efficiency and biological effects of ozonation.

4.1.2 Zerbrafish behavior

Behavioral studies of fish exposed to ozonated STP effluents has not previously been reported, and is thusly a contribution to the field. The novel tank test indicated that fish exposed to ozonated STP effluent displayed an anxity-like response as compared to the tap water control and conventionally treated STP effluent.

A 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 (Bonga, 1997). Stressed fish exhibit induced levels of the stress 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 (Somoza et al., 1988).

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., 2017). Whether these effects would cause adverse impacts on wild fish populations is difficult to predict.

4.1.3 Effects in zebrafish embryos

The embryotoxicity tests performed using embryos and water from Knivsta STP were simplified as to only record proportions of dead and grossly malformed individuals. This method was used due to constraints of time. In Paper II, the embryotoxicity tests were expanded to include more sensitive endpoints (e.g. heart rate).

No significant alterations of deaths and gross malformations between the treatments were found. Furthermore, the larvae locomotion test did not reveal any differences in activity between the treatment groups. Larvae locomotor activity has been used as a sublethal endpoint for assessing neurobehavioral effects of chemicals in the developing fish embryo and larva (MacPhail et al., 2009). Several different classes of suspected neurotoxic chemicals have been screened using this method (Ellis et al., 2012; Ulhaq et al., 2013). In Paper I, such effects were however not detected in embryos exposed to the three treatment groups.

4.2 Ozonation of select pharmaceuticals (paper II)

The biological outcome of the study presented in Paper I was resulting from exposure to a mixture of at least 24 active pharmaceutical ingredients. In order to isolate the contributions of different pharmaceuticals to the toxic response of ozonation, we proceeded in Paper II to test embryotoxicity of three pharmaceuticals from Paper I. These particular pharmaceuticals (carbamazepine, diclofenac, and oxazepam) were chosen because they cover three different classes of drugs, 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 in order to clarify ozone removal efficiencies of the pharmaceuticals in the bench ozonation set-up, as well as investigate the formation of four putative carbamazepine OBPs.

4.2.1 Ozone removal efficiencies of carbamazepine, diclofenac and oxazepam

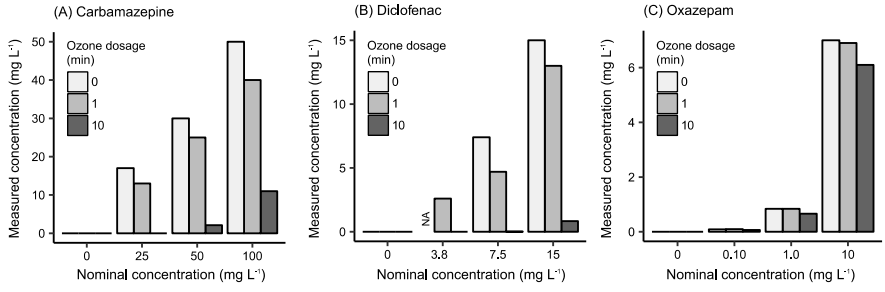

Figure 6. Ozone removal efficiency of (A) carbamazepine, (B) diclofenac and (C) oxazepam after 0, 1 and 10-minute ozonation. The lowest concentration of diclofenac (3.8 mg/L nominal concentration) was not analyzed (NA) due to sampling error. Figure adapted from Paper II.

The achieved ozone removal efficiencies of the studied pharmaceuticals are shown in Figure 6. 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 figures 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 Carbamazepine embryotoxicity

Carbamazepine is a drug used for the treatment of epilepsy and related symptoms. Carbamazepine exposure resulted in embryotoxicity at 144 hpf in the medium (50 mg/L) and highest (100 mg/L) treatment groups with 88% and 100% affected individuals respectively (Figure 7A), mostly due to occurrence of pericardial edema. Carbamazepine also significantly reduced the heart rate at 48 hpf in the highest (100 mg/L) treatment group (Figure 8A). All individuals in the highest (100 mg/L) treatment group were dead prior to hatching (Figure

10A). 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 was a delayed onset of hatching (72 hpf, EC_{50} 45.5 mg/L) and pericardial edema (96 hpf , EC_{50} 52 mg/L). Besides embryotoxicity in zebrafish, carbamazepine has also been shown to cause reproductive toxicity in exposed (100 µg/L) Chinese rare minnows (*Gobiocypris rarus*; Yan et al., (2018)). Since carbamazepine is a highly persistent pharmaceutical in the aquatic environment (e.g. Björlenius et al., 2018), its removal from STP effluents should be considered a priority. 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 (Figures 7A, 8A, 10A). The lowest observed effect concentration (LOEC) of carbamazepine based on percent affected was reduced from the medium concentration (50 mg/L) to the lowest (25 mg/L) following ozonation for 10 min (Figure 7A) The heart rate (Figure 8A) and hatching time (Figure 10A) 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). To our knowledge, there are no other studies reporting zebrafish embryotoxicity of ozonated carbamazepine to date. The cause behind increased toxicity of ozonated carbamazepine was most likely due to the formation of OBPs, which was further investigated in the chemical screening part of Paper II.

4.2.3 Diclofenac embryotoxicity

Diclofenac is a common nonsteroidal anti-inflammatory drug for the treatment of inflammation and related pain. Exposure at medium (7.5 mg/L) and high (15 mg/L) diclofenac concentrations caused pericardial- and yolk-sac edemas, as well as absent systemic circulation, at 48 hpf (Figure 7B). The highest concentration (15 mg /L) exposure also caused reduced heart rate by ~50% (Figure 8B). Subsequently, this treatment group exhibited 100% mortalities at 144 hpf (Figure 7B). Hatching was not affected in the low (3.8 mg/L) and medium (7.5 mg/L) concentration treatment groups, while the highest (15 mg/L)

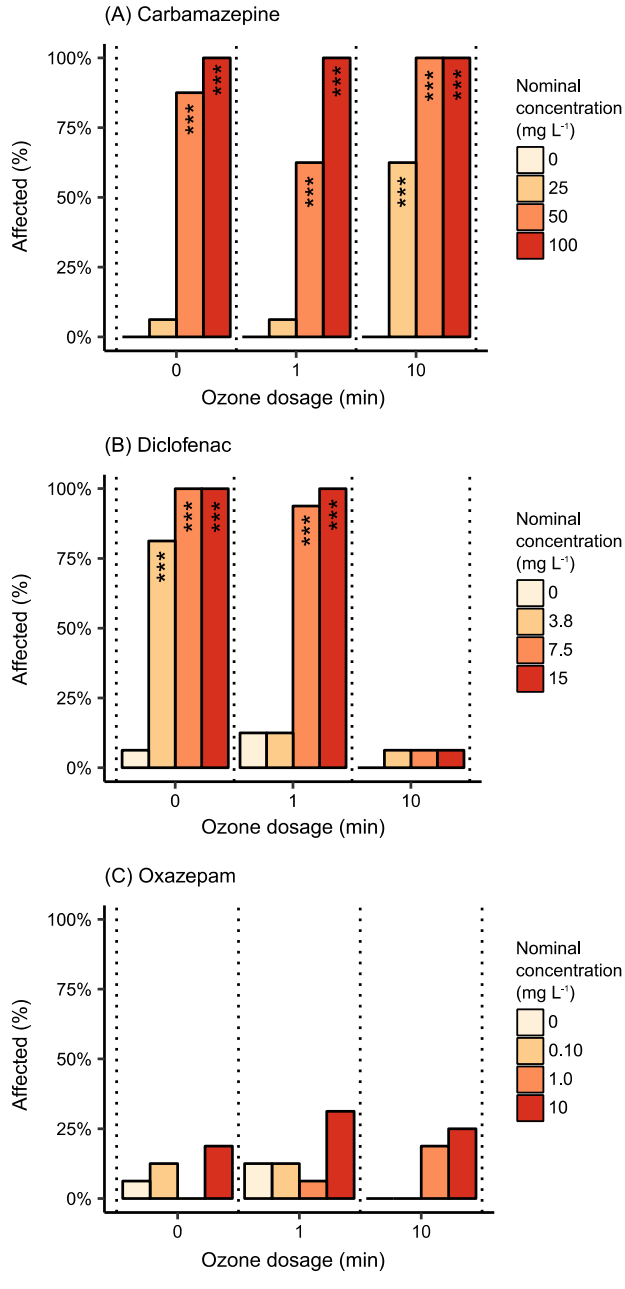

Figure 7. Affected (%, mortalities, and malformations combined) zebrafish embryos at 144 hpf. Significant differences (***: $p<0.001$) between treatments and control were detected for carbamazepine and diclofenac (Bonferroni adjusted Fisher's exact test). Figure adapted from Paper II.

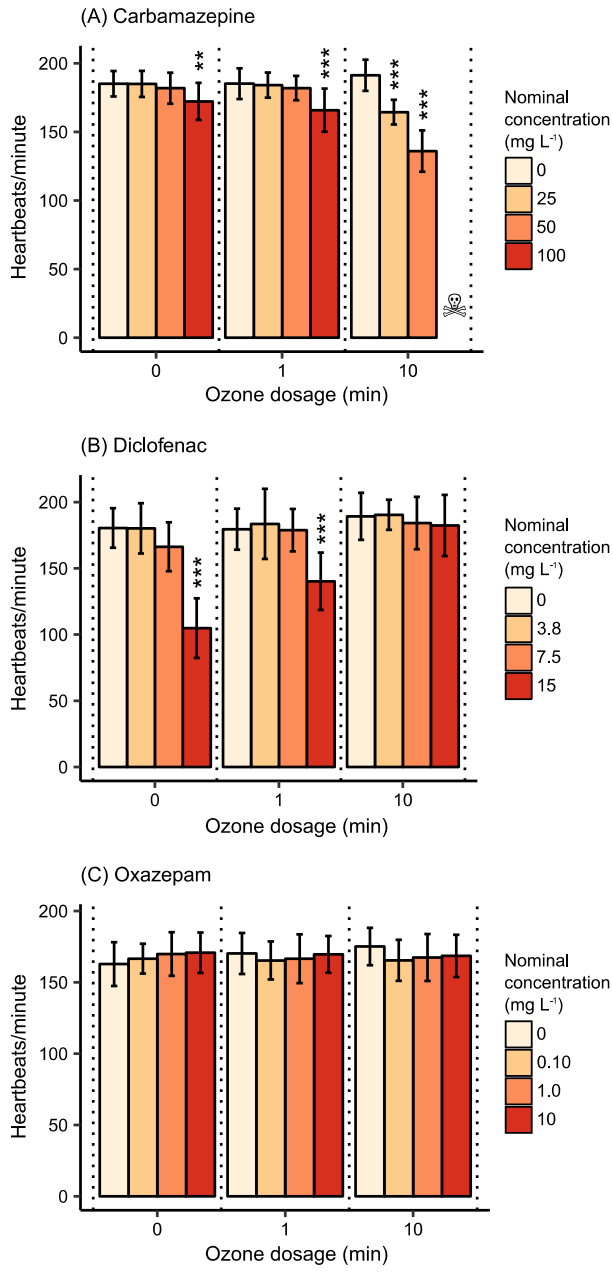

Figure 8. Heart rate (heartbeats/minute, mean ± sd) in zebrafish embryos at 48 hpf. Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test (**: $p<0.01$, ***: $p<0.001$). Figure adapted from Paper II.

concentration group failed to hatch due to 100% pre-hatch mortalities (Figure 10B) 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 control group level after 1 min ozonation (Figure 7B). Diclofenac ozonated for 10 min did not produce any toxicity in any endpoint.

A Microtox (*Vibrio fischeri* NRRL B-11177 strain) *in vitro* study 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. We may conclude that diclofenac ozonation did not form toxic OBP, based on Paper II.

4.2.4 Oxazepam embryotoxicity

Oxazepam is an anxiolytic psychoactive drug used to treat anxiety disorders. In the present study, no alterations in zebrafish lethal or sublethal endpoints were detected in any treatment group, either pre- or post ozonation (Figure 7C, Figure 8C). The larvae locomotor activity screening did not show any deviations in behavior between the different concentrations (Figure 11A). However, ozonation of the highest ozone concentration (10 mg/L) at 10 min caused hypoactivity during dark conditions (Figure 11C).

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.

Only the highest concentration (10 mg/L) of oxazepam treated with the highest dosage of ozone gave rise to the anxiolytic effect. This concentration is considerably higher than concentrations known to produce behavioral effects (910 µg/L, increased boldness) in adult/juvenile roach (Brodin et al., 2013). Embryonal life stages of fish seem to be less sensitive to oxazepam exposure than adult fish. A reduced swimming activity during dark periods has been recorded in larval Japanese medaka exposed to 10 mg/L oxazepam (Chiffre et al., 2016).

The results of Paper II indicated that ozonation of oxazepam at high concentrations (10 mg/L) has the potential to affect fish embryo behavior, which may lead to effects on subsequent survivability.

4.2.5 Carbamazepine OBP formation

The results from the embryotoxicity study indicated how carbamazepine ozonation induced toxicity, possibly by formation of OBPs. This scenario was investigated further by quantifying the putative carbamazepine OBPs Carbamazepine-10,11-epoxide (Cbz-Ep); 10,11-dihydrocarbamazepine (Di-Cbz); 10,11-dihydro-10-hydroxycarbamazepine (DiOH-Cbz), and oxcarbazepine (Ox-Cz). These four were selected due to them being commercially available as reference standards. The formation of these are shown in Figure 9.

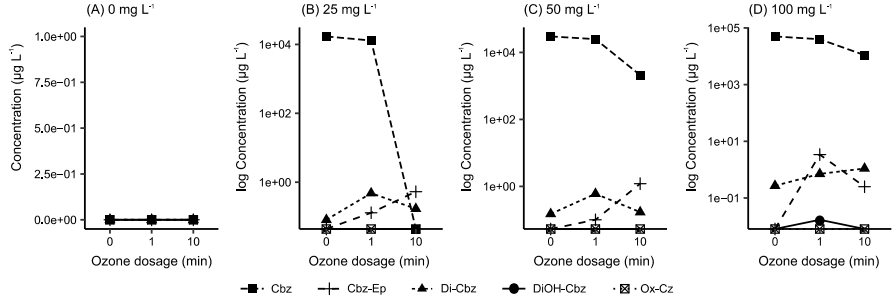

Figure 9. Formation of suspected carbamazepine (Cbz) OBPs (µg/L; Carbamazepine-10,11-epoxide (Cbz-Ep); 10,11-dihydrocarbamazepine (Di-Cbz); 10,11-dihydro-10-hydroxycarbamazepine (DiOH-Cbz), and oxcarbazepine (Ox-Cz)) in the different nominal carbamazepine concentrations (0, 25, 50, 100 mg/L) in dependence on the ozonation dosage. Figure adapted from Paper II.

Cbz-Ep was detected in all ozonated carbamazepine samples, formed increasingly in relation to increasing ozone dosage (Figure 7). It is thus likely that Cbz-Ep contributed significantly to post-ozonated carbamazepine toxicity. 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 is also readily formed by ozonation. In a previous study comparing the

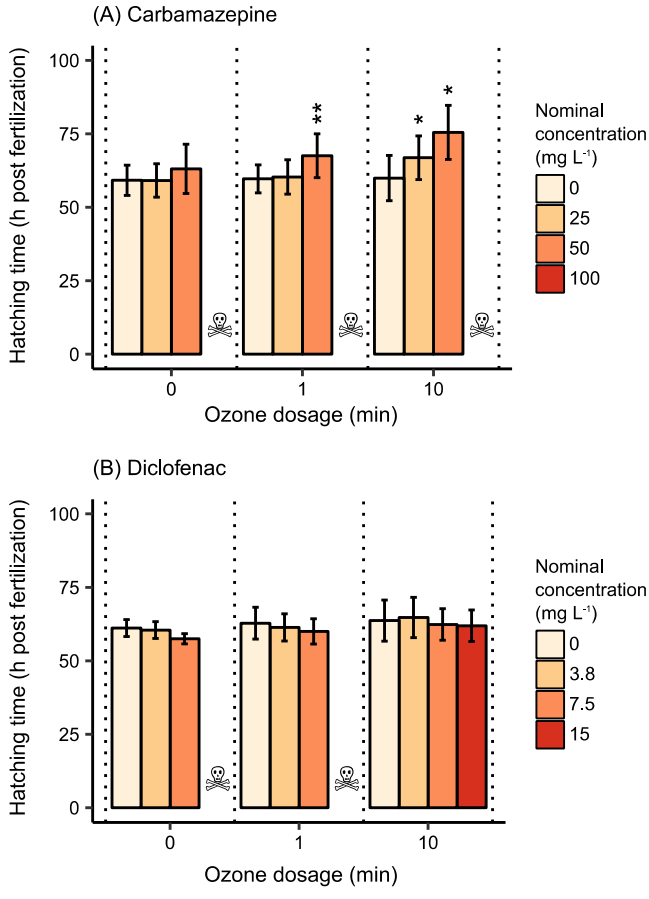

Figure 10. Hatching time (hpf, mean ± sd) of zebrafish embryos exposed to (A) carbamazepine and (B) diclofenac (oxazepam hatching time was not recorded due to technical issues). Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test (*: $p<0.05$, **: $p<0.01$). Figure adapted from Paper II.

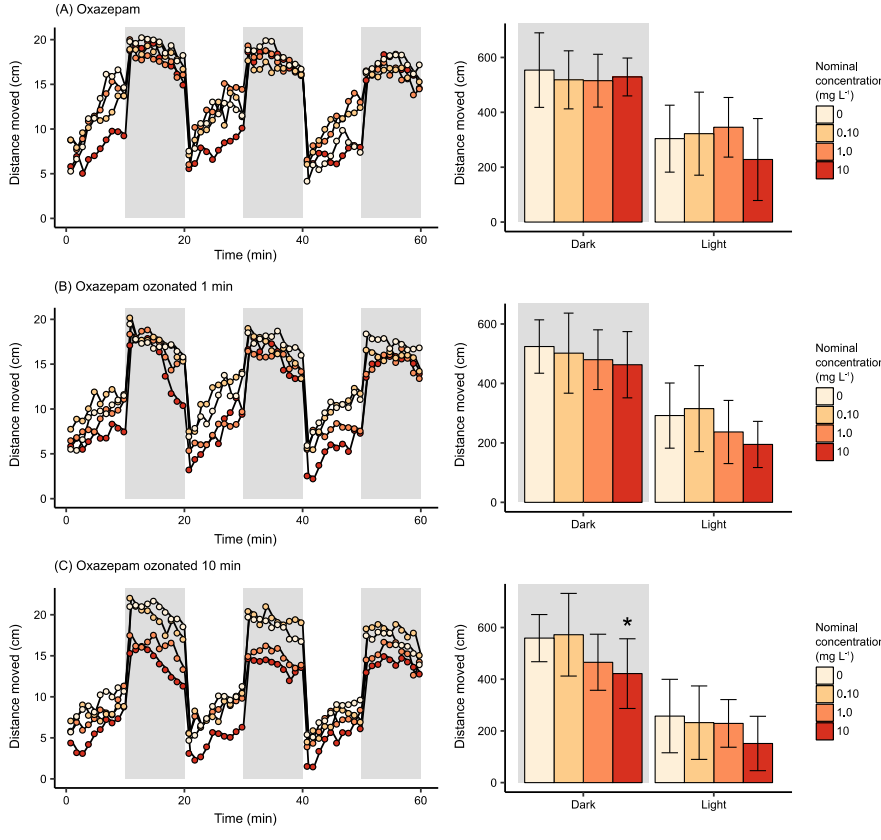

Figure 11. Locomotor activity (distance moved over time) in zebrafish embryos exposed to oxazepam ozonated 0 minutes (A), 1 minute (B) and 10 minutes (C). The left panel shows total distance moved per minute (mean) and the right panel shows mean total distance moved during dark and light conditions (mean±sd). Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test (*: $p<0.05$), considering total distance moved during darkness and light for each individual as a replicate. Figure adapted from Paper II.

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 abundantly detected carbamazepine OBP in Paper II was Di-Cbz. All carbamazepine treatment samples, ozonated and not ozonated, contained concentrations of Di-Cbz exceeding LOQ (Figure 9). This could implicate the action of some metabolizing process independent of ozonation. Carbamazepine metabolites may be formed following treatment in conventional sewage treatment plants, presumably by biotic and abiotic processes (Leclercq et al., 2009).

DiOH-Cbz was only detected (0.017 µg L^{-1}) in the highest carbamazepine concentration ozonated for 1 min. 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 from ozonated carbamazepine.

Increased toxicity of carbamazepine following ozonation, mainly due to the formation of the OBP Cbz-Ep, should be taken into account when considering sewage water ozonation. 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 may affect toxicity outcome in further studies.

5 Conclusion & Future Perspectives

The results presented within this licentiate thesis emphasize the complexity of applying ozonation as an effluent treatment technology due to the formation of toxic OBPs. In Paper I, ozonation of the STP effluent did not cause any major adverse effects in the exposed zebrafish. On the contrary, fish showed a higher fecundity. Yet some unexpected effects of vitellogenin induction and an anxiety-like stress response was observed in these fish as well, indicating unknown processes at this particular STP ozonation facility.

In Paper II, three pharmaceuticals detected in the Knivsta STP effluent were ozonated in a lab-scale ozonation set-up and tested for zebrafish embryotoxicity. Diclofenac emrbyotoxicity was eliminated completely after ozonation. Carbamazepine ozonation increased embryotoxicity, possibly related to the formation of three quantified OBPs. Furthermore, oxazepam produced behavioral effects in zebrafish larvae only after ozonation. It is, however, important to highlight that the embryotoxicity studies presented in Paper II used pharmaceutical concentrations far above what is normally found in STP effluents. This was a matter of sensitivity in the selected endpoints since exposure at lower, environmentally relevant, concentrations would not cause significant responses. Paper II should, therefore, be viewed as an experimental investigation into the embryotoxic outcomes of pharmaceutical ozonation.

Ozonation of STP effluents has been shown to be a complex undertaking, manifesting both beneficial and potentially adverse outcomes. In order to reach satisfactory pharmaceutical residue elimination without risking increased toxicity, the method must be designed and operated with caution. Further research which investigates the identities and toxicities of OBPs is needed, as well as methods to prevent the formation of or further degrade these compounds.

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References

Albertsson, E., Larsson, D.G.J., Förlin, L., 2010. Induction of hepatic carbonyl reductase/20β-hydroxysteroid dehydrogenase mRNA in rainbow trout downstream from sewage treatment works—Possible roles of aryl hydrocarbon receptor agonists and oxidative stress. Aquatic Toxicology, Genomics Issue 97, 243–249. https://doi.org/10.1016/j.aquatox.2009.11.007

aus der Beek, T., Weber, F.-A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küster, A., 2016. Pharmaceuticals in the environment-Global occurrences and perspectives: Pharmaceuticals in the global environment. Environmental Toxicology and Chemistry 35, 823–835. https://doi.org/10.1002/etc.3339

Beijer, K., Björlenius, B., Shaik, S., Lindberg, R.H., Brunström, B., Brandt, I., 2017. Removal of pharmaceuticals and unspecified contaminants in sewage treatment effluents by activated carbon filtration and ozonation: Evaluation using biomarker responses and chemical analysis. Chemosphere 176, 342–351. https://doi.org/10.1016/j.chemosphere.2017.02.127

Beker van Woudenberg, A., Snel, C., Rijkmans, E., de Groot, D., Bouma, M., Hermsen, S., Piersma, A., Menke, A., Wolterbeek, A., 2014. Zebrafish embryotoxicity test for developmental (neuro)toxicity: Demo case of an integrated screening approach system using anti-epileptic drugs. Reproductive Toxicology 49, 101–116. https://doi.org/10.1016/j.reprotox.2014.07.082

Bila, D., Montalvão, A.F., Azevedo, D. de A., Dezotti, M., 2007. Estrogenic activity removal of 17β-estradiol by ozonation and identification of by-products. Chemosphere 69, 736–746. https://doi.org/10.1016/j.chemosphere.2007.05.016

Björkblom, C., Högfors, E., Salste, L., Bergelin, E., Olsson, P.-E., Katsiadaki, I., Wiklund, T., 2009. Estrogenic and androgenic effects of municipal wastewater effluent on reproductive endpoint biomarkers in three-spined stickleback (Gasterosteus aculeatus). Environmental

Toxicology and Chemistry 28, 1063–1071. https://doi.org/10.1897/08-337.1

Björlenius, B., Ripszám, M., Haglund, P., Lindberg, R.H., Tysklind, M., Fick, J., 2018. Pharmaceutical residues are widespread in Baltic Sea coastal and offshore waters – Screening for pharmaceuticals and modelling of environmental concentrations of carbamazepine. Science of The Total Environment 633, 1496–1509. https://doi.org/10.1016/j.scitotenv.2018.03.276

Bonga, S.E.W., 1997. The stress response in fish. Physiological Reviews 77, 591–625.

Breton, H., Cociglio, M., Bressolle, F., Peyriere, H., Blayac, J.P., Hillaire-Buys, D., 2005. Liquid chromatography–electrospray mass spectrometry determination of carbamazepine, oxcarbazepine and eight of their metabolites in human plasma. Journal of Chromatography B 828, 80–90. https://doi.org/10.1016/j.jchromb.2005.09.019

Brian, J.V., Harris, C.A., Scholze, M., Kortenkamp, A., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Marcomini, A., Sumpter, J.P., 2007. Evidence of Estrogenic Mixture Effects on the Reproductive Performance of Fish. Environ. Sci. Technol. 41, 337–344. https://doi.org/10.1021/es0617439

Brodin, T., Fick, J., Jonsson, M., Klaminder, J., 2013. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science 339, 814–815. https://doi.org/10.1126/science.1226850

Burgess, H.A., Granato, M., 2007. Modulation of locomotor activity in larval zebrafish during light adaptation. Journal of Experimental Biology 210, 2526–2539. https://doi.org/10.1242/jeb.003939

Chiffre, A., Clérandeau, C., Dwoinikoff, C., Bihanic, F.L., Budzinski, H., Geret, F., Cachot, J., 2016. Psychotropic drugs in mixture alter swimming behaviour of Japanese medaka (*Oryzias latipes*) larvae above environmental concentrations. Environ Sci Pollut Res 23, 4964–4977. https://doi.org/10.1007/s11356-014-3477-4

Coelho, A.D., Sans, C., Agüera, A., Gómez, M.J., Esplugas, S., Dezotti, M., 2009. Effects of ozone pre-treatment on diclofenac: Intermediates, biodegradability and toxicity assessment. Science of The Total Environment 407, 3572–3578. https://doi.org/10.1016/j.scitotenv.2009.01.013

Cuklev, F., Gunnarsson, L., Cvijovic, M., Kristiansson, E., Rutgersson, C., Björlenius, B., Larsson, D.G.J., 2012. Global hepatic gene expression in rainbow trout exposed to sewage effluents: A comparison of different sewage treatment technologies. Science of The Total Environment 427–428, 106–114. https://doi.org/10.1016/j.scitotenv.2012.04.009

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ Health Perspect 107, 907–938.
de Wilt, A., van Gijn, K., Verhoek, T., Vergnes, A., Hoek, M., Rijnaarts, H., Langenhoff, A., 2018. Enhanced pharmaceutical removal from water in a three step bio-ozone-bio process. Water Research 138, 97–105. https://doi.org/10.1016/j.watres.2018.03.028
Dwivedi, K., Rudrashetti, A.P., Chakrabarti, T., Pandey, R.A., 2018. Transformation products of carbamazepine (CBZ) after ozonation and their toxicity evaluation using *Pseudomonas* sp. Strain KSH-1 in aqueous matrices. Indian J Microbiol 58, 193–200. https://doi.org/10.1007/s12088-018-0715-3
Eggen, R.I.L., Hollender, J., Joss, A., Schärer, M., Stamm, C., 2014. Reducing the Discharge of Micropollutants in the Aquatic Environment: The Benefits of Upgrading Wastewater Treatment Plants. Environ. Sci. Technol. 48, 7683–7689. https://doi.org/10.1021/es500907n
Ellis, L.D., Seibert, J., Soanes, K.H., 2012. Distinct models of induced hyperactivity in zebrafish larvae. Brain Research 1449, 46–59. https://doi.org/10.1016/j.brainres.2012.02.022
Escher, B.I., Fenner, K., 2011. Recent Advances in Environmental Risk Assessment of Transformation Products. Environ. Sci. Technol. 45, 3835–3847. https://doi.org/10.1021/es1030799
European Union, 2015. Commission Implementing Decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council (notified under document C(2015) 1756). Off. J. Eur. Union, L (78/40).
Gunnarsson, L., Adolfsson-Erici, M., Björlenius, B., Rutgersson, C., Förlin, L., Larsson, D.G.J., 2009. Comparison of six different sewage treatment processes—Reduction of estrogenic substances and effects on gene expression in exposed male fish. Science of The Total Environment 407, 5235–5242. https://doi.org/10.1016/j.scitotenv.2009.06.018
Han, Y., Ma, M., Li, N., Hou, R., Huang, C., Oda, Y., Wang, Z., 2018. Chlorination, chloramination and ozonation of carbamazepine enhance cytotoxicity and genotoxicity: Multi-endpoint evaluation and identification of its genotoxic transformation products. Journal of Hazardous Materials 342, 679–688. https://doi.org/10.1016/j.jhazmat.2017.08.076
Heye, K., Becker, D., Lütke Eversloh, C., Durmaz, V., Ternes, T.A., Oetken, M., Oehlmann, J., 2016. Effects of carbamazepine and two of its metabolites on the non-biting midge Chironomus riparius in a sediment full life cycle toxicity test. Water Research 98, 19–27. https://doi.org/10.1016/j.watres.2016.03.071

Hill, A.J., Teraoka, H., Heideman, W., Peterson, R.E., 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci 86, 6–19. https://doi.org/10.1093/toxsci/kfi110

Hughes, S.R., Kay, P., Brown, L.E., 2013. Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems. Environ Sci Technol 47, 661–677. https://doi.org/10.1021/es3030148

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., Baunbeck, T., Turner, A.P., Tyler, C.R., 2003. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquatic Toxicology 65, 205–220. https://doi.org/10.1016/S0166-445X(03)00134-6

Kalueff, A.V., Gebhardt, M., Stewart, A.M., Cachat, J.M., Brimmer, M., Chawla, J.S., Craddock, C., Kyzar, E.J., Roth, A., Landsman, S., Gaikwad, S., Robinson, K., Baatrup, E., Tierney, K., Shamchuk, A., Norton, W., Miller, N., Nicolson, T., Braubach, O., Gilman, C.P., Pittman, J., Rosemberg, D.B., Gerlai, R., Echevarria, D., Lamb, E., Neuhauss, S.C.F., Weng, W., Bally-Cuif, L., Schneider, H., 2013. Towards a Comprehensive Catalog of Zebrafish Behavior 1.0 and Beyond. Zebrafish 10, 70–86. https://doi.org/10.1089/zeb.2012.0861

Larsson, D.G.J., 2014. Pollution from drug manufacturing: review and perspectives. Philos Trans R Soc Lond B Biol Sci 369. https://doi.org/10.1098/rstb.2013.0571

Larsson, D.G.J., de Pedro, C., Paxeus, N., 2007. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. Journal of Hazardous Materials 148, 751–755. https://doi.org/10.1016/j.jhazmat.2007.07.008

Lavén, M., Alsberg, T., Yu, Y., Adolfsson-Erici, M., Sun, H., 2009. Serial mixed-mode cation- and anion-exchange solid-phase extraction for separation of basic, neutral and acidic pharmaceuticals in wastewater and analysis by high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry. Journal of Chromatography A 1216, 49–62. https://doi.org/10.1016/j.chroma.2008.11.014

Leclercq, M., Mathieu, O., Gomez, E., Casellas, C., Fenet, H., Hillaire-Buys, D., 2009. Presence and fate of carbamazepine, oxcarbazepine, and seven of their metabolites at wastewater treatment plants. Arch Environ Contam Toxicol 56, 408. https://doi.org/10.1007/s00244-008-9202-x

Lee, Y., von Gunten, U., 2016. Advances in predicting organic contaminant abatement during ozonation of municipal wastewater effluent: reaction kinetics, transformation products, and changes of biological effects. Environ. Sci.: Water Res. Technol. 2, 421–442. https://doi.org/10.1039/C6EW00025H

Liney, K.E., Hagger, J.A., Tyler, C.R., Depledge, M.H., Galloway, T.S., Jobling, S., 2005. Health Effects in Fish of Long-Term Exposure to

Effluents from Wastewater Treatment Works. Environmental Health Perspectives 114, 81–89. https://doi.org/10.1289/ehp.8058
Lister, A., Regan, C., Van Zwol, J., Van Der Kraak, G., 2009. Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: A mechanistic evaluation. Aquatic Toxicology, Zebrafish Issue 95, 320–329. https://doi.org/10.1016/j.aquatox.2009.04.011
Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262
Lundström, E., Adolfsson-Erici, M., Alsberg, T., Björlenius, B., Eklund, B., Lavén, M., Breitholtz, M., 2010. Characterization of additional sewage treatment technologies: Ecotoxicological effects and levels of selected pharmaceuticals, hormones and endocrine disruptors. Ecotoxicology and Environmental Safety 73, 1612–1619. https://doi.org/10.1016/j.ecoenv.2010.05.012
Ma, T., Wan, X., Huang, Q., Wang, Z., Liu, J., 2005. Biomarker responses and reproductive toxicity of the effluent from a Chinese large sewage treatment plant in Japanese medaka (Oryzias latipes). Chemosphere 59, 281–288. https://doi.org/10.1016/j.chemosphere.2004.11.033
MacPhail, R.C., Brooks, J., Hunter, D.L., Padnos, B., Irons, T.D., Padilla, S., 2009. Locomotion in larval zebrafish: Influence of time of day, lighting and ethanol. NeuroToxicology 30, 52–58. https://doi.org/10.1016/j.neuro.2008.09.011
Malaj, E., Ohe, P.C. von der, Grote, M., Kühne, R., Mondy, C.P., Usseglio-Polatera, P., Brack, W., Schäfer, R.B., 2014. Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. PNAS 111, 9549–9554. https://doi.org/10.1073/pnas.1321082111
Maletz, S., Floehr, T., Beier, S., Klümper, C., Brouwer, A., Behnisch, P., Higley, E., Giesy, J.P., Hecker, M., Gebhardt, W., Linnemann, V., Pinnekamp, J., Hollert, H., 2013. In vitro characterization of the effectiveness of enhanced sewage treatment processes to eliminate endocrine activity of hospital effluents. Water Research 47, 1545–1557. https://doi.org/10.1016/j.watres.2012.12.008
Maya, N., Evans, J., Nasuhoglu, D., Isazadeh, S., Yargeau, V., Metcalfe, C.D., 2017. Evaluation of wastewater treatment by ozonation for reducing the toxicity of contaminants of emerging concern to rainbow trout (Oncorhynchus mykiss). Environ Toxicol Chem. https://doi.org/10.1002/etc.3952
Nichols, K.M., Miles-Richardson, S.R., Snyder, E.M., Giesy, J.P., 1999. Effects of exposure to municipal wastewater in situ on the reproductive physiology of the fathead minnow (Pimephales promelas). Environmental Toxicology and Chemistry 18, 2001–2012. https://doi.org/10.1002/etc.5620180919

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, 93–101. https://doi.org/10.1016/j.aquatox.2018.04.014

Purdom, C.E., Hardiman, P.A., Bye, V.V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P., 1994. Estrogenic Effects of Effluents from Sewage Treatment Works. Chemistry and Ecology 8, 275–285. https://doi.org/10.1080/02757549408038554

R Core team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rose, E., Paczolt, K.A., Jones, A.G., 2013. The effects of synthetic estrogen exposure on premating and postmating episodes of selection in sex-role-reversed Gulf pipefish. Evol Appl 6, 1160–1170. https://doi.org/10.1111/eva.12093

Routledge, E.J., Sheahan, D., Desbrow, C., Brighty, G.C., Waldock, M., Sumpter, J.P., 1998. Identification of Estrogenic Chemicals in STW Effluent. 2. In Vivo Responses in Trout and Roach. Environ. Sci. Technol. 32, 1559–1565. https://doi.org/10.1021/es970796a

RStudio Team, 2016. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA.

Samuelsson, L.M., Björlenius, B., Förlin, L., Larsson, D.G.J., 2011. Reproducible ^{1}H NMR-Based Metabolomic Responses in Fish Exposed to Different Sewage Effluents in Two Separate Studies. Environmental Science & Technology 45, 1703–1710. https://doi.org/10.1021/es104111x

Somoza, G.M., Yu, K.L., Peter, R.E., 1988. Serotonin stimulates gonadotropin release in female and male goldfish, Carassius auratus L. Gen. Comp. Endocrinol. 72, 374–382.

Sun, J., Wang, J., Zhang, R., Wei, D., Long, Q., Huang, Y., Xie, X., Li, A., 2017. Comparison of different advanced treatment processes in removing endocrine disruption effects from municipal wastewater secondary effluent. Chemosphere 168, 1–9. https://doi.org/10.1016/j.chemosphere.2016.10.031

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Research 32, 3245–3260. https://doi.org/10.1016/S0043-1354(98)00099-2

Thorpe, K.L., Maack, G., Benstead, R., Tyler, C.R., 2009. Estrogenic Wastewater Treatment Works Effluents Reduce Egg Production in Fish. Environ. Sci. Technol. 43, 2976–2982. https://doi.org/10.1021/es803103c

Ulhaq, M., Örn, S., Carlsson, G., Morrison, D.A., Norrgren, L., 2013. Locomotor behavior in zebrafish (*Danio rerio*) larvae exposed to perfluoroalkyl acids. Aquatic Toxicology 144–145, 332–340. https://doi.org/10.1016/j.aquatox.2013.10.021

Valdés, M.E., Huerta, B., Wunderlin, D.A., Bistoni, M.A., Barceló, D., Rodriguez-Mozaz, S., 2016. Bioaccumulation and bioconcentration of carbamazepine and other pharmaceuticals in fish under field and controlled laboratory experiments. Evidences of carbamazepine metabolization by fish. Science of The Total Environment 557–558, 58–67. https://doi.org/10.1016/j.scitotenv.2016.03.045

van Aerle, R., Nolanusan, M., Jobling, S., Christiansen, L.B., Sumpter, J.P., Tyler, C.R., 2001. Sexual disruption in a second species of wild cyprinid fish (the gudgeon, Gobio gobio) in United Kingdom Freshwaters. Environmental Toxicology and Chemistry 20, 2841–2847. https://doi.org/10.1002/etc.5620201225

von Gunten, U., 2003. Ozonation of drinking water: Part I. Oxidation kinetics and product formation. Water Research 37, 1443–1467. https://doi.org/10.1016/S0043-1354(02)00457-8

Wigh, A., Devaux, A., Brosselin, V., Gonzalez-Ospina, A., Domenjoud, B., Aït-Aïssa, S., Creusot, N., Gosset, A., Bazin, C., Bony, S., 2015. Proposal to optimize ecotoxicological evaluation of wastewater treated by conventional biological and ozonation processes. Environ Sci Pollut Res 23, 3008–3017. https://doi.org/10.1007/s11356-015-5419-1

Winberg, S., Nilsson, G.E., Olsén, K.H., 1992. The Effect of Stress and Starvation on Bratn Serotonin Utilization in Arctic Charr (Salvelinus Alpinus). Journal of Experimental Biology 165, 229–239.

Yan, S., Wang, M., Zha, J., Zhu, L., Li, W., Luo, Q., Sun, J., Wang, Z., 2018. Environmentally relevant concentrations of carbamazepine caused endocrine-disrupting effects on nontarget organisms, Chinese Rare Minnows (*Gobiocypris rarus*). Environ. Sci. Technol. 52, 886–894. https://doi.org/10.1021/acs.est.7b06476

Zimmermann, S.G., Wittenwiler, M., Hollender, J., Krauss, M., Ort, C., Siegrist, H., von Gunten, U., 2011. Kinetic assessment and modeling of an ozonation step for full-scale municipal wastewater treatment: Micropollutant oxidation, by-product formation and disinfection. Water Research 45, 605–617. https://doi.org/10.1016/j.watres.2010.07.080

Ågerstrand, M., Berg, C., Björlenius, B., Breitholtz, M., Brunström, B., Fick, J., Gunnarsson, L., Larsson, D.G.J., Sumpter, J.P., Tysklind, M., Rudén, C., 2015. Improving Environmental Risk Assessment of Human Pharmaceuticals. Environmental Science & Technology 49, 5336–5345. https://doi.org/10.1021/acs.est.5b00302

Popular science summary

My research has examined ozonation as a sewage treatment technology. Ozone is a very potent oxidizer which efficiently breaks down molecules such as pharmaceuticals. The end goal of sewage ozonation is to improve the aquatic environment, and protect drinking water sources, by removing pharmaceuticals. But what about the biological effects in fish exposed to ozonated sewage water? Since the current state of Europe's freshwater environments is under threat it is important to make sure that ozone will not create a worse scenario.

This licentiate thesis presents two studies on fish; the first one investigating the biological effects on fish swimming in ozonated sewage water and the second shows the impact of ozonation on the embryotoxicity of the commonly used pharmaceuticals diclofenac, carbamazepine, and oxazepam.

Medicines and drugs are of immense use for the health and welfare of human and animal patients alike. Medicine prolongs our lives and cures us of painful diseases. The production and sale of pharmaceuticals are multi-billion dollar industries which are set to grow considerably as the need and demand for medicine continue to grow in a growing world market. But what happens with the medicine we eat or give to our animals when it exits out into the sewage systems?

Pharmaceuticals pass through and exit our bodies in urine and faeces either completely or partly metabolized. Consequently, they end up in the environment via our sewage systems. Sewage treatment plants have a great potential of removing nitrogen and phosphorous from incoming sewage water. This prevents environmental damage in the form of eutrophication, which is a huge threat to the aquatic environment, especially in the Baltic Sea. However, at this point pharmaceuticals easily drifts through the sewage treatment plants where they have a potential to seriously disturb natural populations of fish and other aquatic animals.

What could be done to reduce and remove these harmful substances from the environment? This is where ozone sewage water treatment could play a part.

Ozone has been used for the purpose of sewage treatment for a long time, but its full potential as a pharmaceutical removal has never really caught on until recent years. It has been established in many studies to be an incredibly effective removal agent of pharmaceuticals.

The pharmaceutical removal capacity of ozone has been established but what about the biological effects in animals exposed to ozonated sewage water? There are more than 30 studies published in peer-reviewed journals that reports data from studies comparing different biological responses, or endpoints, in fish exposed to either normally treated sewage and ozonated sewage. They show mostly promising results but also some potential risks. Most of the research has been done in Europe where some countries, including Sweden and Switzerland, already have started using ozonation in existing sewage treatment plants.

During the winter months of 2015 to 2016, a full-scale ozonation of Knivsta municipal sewage treatment plant was performed as part of a project funded by Havs och vattenmyndigheten. SLU, KTH and Umeå University were part of this project. KTH was responsible for the construction and operation of the ozonation step. Umeå University assessed the ecological status in the Knivsta River prior, during and after the ozonation and me and my colleagues at SLU designed and operated an experimental container with aquariums connected to both ozonated and not ozonated sewage effluent.

Our results from the first study showed that zebrafish exposed to ozonated water spawned twice as many eggs as the non-ozonated and tap water treatments. Whether or not the increased production of eggs in the ozonated treatment group should be classified as a negative or beneficial effect is probably up for debate. Interestingly, the increase of egg production in fish exposed to ozonated sewage has also been observed in the 1970's by researchers in the United States. The fish exposed to the ozonated effluent were also seemingly stressed. What caused the stress this is however unknown, but could be very interesting to look further into.

The sewage water is a cocktail of pharmaceutical residues and other pollution, and it is a challenge to find out what causes what response in the exposed fish. Therefore we continued studying the impact of ozonation on the embryotoxicity of specific pharmaceuticals for the second study. Diclofenac (Sold with the trade name Voltaren®, an anti-inflammatory painkiller) carbamazepine (an anti-epileptic drug) and oxazepam (an anxiolytic drug) were chosen for this purpose, since they are commonly used and found in sewage and the environment.

The results showed that ozone can be both good and bad, a double-edged sword in this context. It gave rise to increased toxicity of carbamazepine and oxazepam, but eliminated all toxicity of diclofenac. The reason why this

treatment is effective in the first place is due to the reactive ability of ozone. It interacts with other molecules, in best cases shattering them apart. The negative effects could be, as the results indicated, that other more harmful compounds are formed. These compounds are partially degraded and kind of mimics pharmaceuticals which have been transformed in the liver, after a patient has swallowed the pill. Therefore, ozone may turn ‘inactive’ pharmaceuticals into ‘super-active’ pharmaceuticals.

These results add on to the complexity of ozonation as a sewage treatment technology, implicating the need to properly evaluate the effects of ozonation further. As mentioned earlier, many countries and cities have already started to implement ozone treatment in existing sewage treatment plants. It will remain important to continue working to find ways of improving sewage water treatment, in order to prevent harmful effects in aquatic organisms, and keep the water environment clean for future generations. Maybe, ozonation could be one answer, if done right.

Populärvetenskaplig sammanfattning

Min forskning har undersökt ozonering som en metod för avloppsrening. Ozon är en mycket kraftfull oxidant som effektivt bryter ner molekyler såsom läkemedel. Målet med att använda ozon vid avloppsrening är att förbättra vattenmiljön samt skydda dricksvattnet genom att avlägsna de läkemedelsrester som i nuläget inte går att få bort. Ozonering ser ut att vara en lovande lösning på problemet men hur påverkas den fisk som sedan simmar i det ozonerade avloppsvattnet? Eftersom Europas sötvattensmiljöer redan nu är under hot är det av stor vikt att säkerställa att ozon inte skapar en ännu värre situation.

Denna licentiatavhandling bygger på två fiskstudier; den första undersökte de biologiska effekterna på fisk som simmar i ozonerat avloppsvatten och den andra fokuserar på hur ozonering påverkar embryots dödlighet i relation till tre läkemedel som vanligtvis går att hitta i vårt avloppsvatten: diklofenak, karbamazepin och oxazepam.

Mediciner och läkemedel är utan tvekan av oerhörd nytta för både människor och djurs hälsa och välmående. Tack vare läkemedel förlängs våra liv och vi kan botas från smärtsamma sjukdomar. Läkemedelstillverkningen och dess distribution är i nuläget en multi-miljardindustri som fortsätter att växa i takt med att världens befolkning ökar. Men vad händer med den medicin vi äter eller ger våra djur när den senare försvinner iväg ut i avloppet?

Läkemedel tas upp av våra kroppar och kommer sedan ut genom urin och avföring, antingen i hel eller delvis nedbruten form. Tillslut hamnar resterna i miljön via vattnet i våra avloppssystem. Våra avloppsreningsverk kan effektivt få bort kväve och fosfor från avloppsvattnet. Detta förebygger miljöskador i form av övergödning, vilket är stort hot för den akvatiska miljön, särskilt i Östersjön. Men det som inte går att få bort är läkemedelsresternas som i nuläget utan problem färdas genom systemen. När resterna kommer ut är risken stor att de förgiftar eller på andra vis skadar fiskar och andra vattenlevande organismer. Så vad kan göras för att reducera eller få bort dessa skadliga ämnen från miljön? Det är här som vattenrening med hjälp av ozonering skulle kunna vara en

lösning. Ozon har sedan länge används i samband med avloppsrening men det är på senare år dess effektivitet som läkemedelsbortagare har kommit att undersökas närmare. Nu har många studier kunnat påvisa hur otroligt effektivt ozon kan få bort läkemedelsrester från vatten.

Om forskningen i nuläget har kunnat fastställa att ozonering tar bort läkemedelsrester vid avloppsrening så kvarstår frågan kring vilka biologiska effekter man kan se i de vattenlevande organismer som lever i det ozonerade vattnet. Mer än 30 studier publicerade i vetenskapligt granskade tidskrifter rapporterar resultat som visar olika biologiska mått och värden tagna på fisk exponerade för antingen vanligt avloppsvatten eller ozonerat avloppsvatten. Resultaten ser lovande ut men det finns även risker som bör uppmärksammas. Den största delen av forskningen har gjorts i Europa och i några länder, såsom Sverige och Schweiz, har man redan börjat implementera ozonering på existerande avloppsreningsverk.

Under vintern 2015-2016 utfördes ett fullskaligt ozonerings-projekt på Knivsta kommuns avloppsreningsverk. SLU, KTH och Umeå Universitet var en del av försöket som i sin tur var en del av ett större projekt finansierat av Havs och vattenmyndigheten. KTH ansvarade för konstruktion och övervakning av ozonerings processen. Umeå universitet utvärderade den ekologiska statusen i Knivstaån före, under och efter ozonering och jag med kollegor på SLU designade och drev en temporär forskningsstation på plats (i en container) bestående av akvarium kopplade till både ozonerat och icke ozonerat vatten.

Våra resultat från den första studien visade att zebrafisken som simmade i det ozonerade avloppsvattnet la dubbelt som många ägg i jämförelse med de fiskar som simmade i icke ozonerat avloppsvatten samt i kranvatten. Utifall den ökade ägg-produktionen hos fiskarna i det ozonerade vattnet är något som ska tolkas som negativt eller positivt får antagligen "vara upp till debatt". Intressant nog kunde en forskningsgrupp redan på 1970-talet i USA se samma tendenser i likvärdiga försök. Fisken som simmade i det ozonerade avsloppsvattnet var även mer stressad. Vad som orsakade denna stress kan inte min undersökning svara på, men kunde vara mycket intressant att titta närmare på.

I avloppsvattnet finns en blandning av olika läkemedelsrester och andra föroreningar. Det är därför minst sagt en utmaning att identifiera vad det är som orsakar vilken "respons" hos den exponerade fisken. Därför bestämde vi oss för att den andra studien skulle undersöka hur "embryo-dödligheten" står i relation till ozonering och några särskilda läkemedel. Diklofenak (säljs som Voltaren®, en anti-inflammatorisk smärtstillare), karbamazepin (ett anti-epileptiskt läkemedel) och oxazepam (ett ångestdämpande läkemedel) valdes då de är vanligt använda läkemedel som kan hittas i avloppsverken och i miljön. Resultaten visade på att ozonering både kan vara bra och dåligt. Ozoneringen

gjorde så att karbamazepin och oxazepam blev mer skadliga medan i sin tur all skadlighet kopplad till diklofenak försvann. Anledningen till att denna metod är så effektiv ligger i ozonets reaktiva förmåga. När ozon interagerar med andra molekyler bryts de vanligtvis ner och läkemedlet blir i sin tur overksamt. De negativa sidoeffekterna, som resultaten indikerade på, är att andra ännu mer skadliga ämnen i vissa fall kan bildas. Läkemedelsrester som är delvis nedbrutna kan i vissa fall efter ozoneringen börja efterlikna läkemedel som de en gång var och kopplar ihop sig med andra molekyler för att uppnå detta. Det är på detta vis som ozon har förmågan att förvandla inaktiva läkemedel till "superaktiva" läkemedel.

Mina forskningsresultat förstärker ozonets komplexitet som en avloppsvattenreningsmetod och belyser behovet av att vidare undersöka effekterna av ozonering. Som jag nämnde tidigare har redan nu ett flertal länder och städer börjat implementera ozonering på sina existerande avloppsreningsverk. Även i framtiden kommer det vara av största vikt att förbättra våra vattenreningstekniker för att kunna förhindra skadliga effekter hos vattenlevande organismer. Det är även av största vikt att framtida generationer ska kunna få rent dricksvatten vilket hänger ihop med en frisk vattenmiljö. Kanske är ozonering en del av lösningen på problemet, om man hittar rätt sätt att göra det på.