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Biomarker response in rainbow trout *(Oncorhynchus mykiss)* exposed to additionally treated sewage treatment plant effluent

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Contents

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Abstract

The global extensive use of pharmaceuticals has led to a widespread contamination of the environment. After usage the major part of the pharmaceuticals ends up in the sewage and finally in sewage treatment plants. Sewage treatment plants are primarily constructed to remove eutrophicating substances and microbial contaminants but are inefficient in removing many pharmaceuticals. The left over pharmaceuticals are then let out in recipients in high enough concentrations to cause adverse effects in wild fish populations. Furthermore pharmaceuticals are found in drinking water. These findings have urged development of new treatment technologies that are capable of removing pharmaceuticals to a higher degree. In this study two promising treatment methods were evaluated by biological testing. Rainbow trout *(Oncorhynchus mykiss)* were exposed to sewage treatment plant effluent water, ozonated effluent water and effluent water filtered through activated carbon. Fish exposed to tap water served as controls. Gill ethoxyresorufin O-deethylase (EROD) activity was measured along with mRNA transcript analysis in gill and liver of 15 genes involved in xenobiotic metabolism, oxidative stress and estrogen receptor activation. In general activated carbon filtered water showed reduced EROD activity and down regulation of two of the three analyzed cytochrome p450 (CYP) genes and glutathione S-transferase A (GST A) compared to effluent, indicating the ability of activated carbon to remove xenobiotics from the water. Ozonation of the water also resulted in reduced EROD activity along with down regulation of the CYP1A genes in gills. However carbonyl reductase A (CBR A) was slightly induced in gills of fish exposed to ozonated water, indicating incomplete removal of chemicals and/or formation of toxic products.

Keywords: rainbow trout, sewage treatment, pharmaceuticals, gene expression, EROD, oxidative stress, xenobiotic metabolism, estrogen

Introduction

In the 1960s pollution caused by sewage outlets was given a lot of attention in Sweden. Lakes were overgrown by vegetation and algal blooms increased due to eutrophication, mass fish death was caused by anoxic conditions, people got ill while bathing due to microbial contamination and heavy metals were found in lake sediments. In the 1970s large economic efforts were put into building and improving municipal sewage treatment plants (STPs) and giving subsidies to industries to treat their sewage. At that time STPs were mainly constructed to remove eutrophicating substances such as nitrogen and phosphorus, organic substances and microbial contamination. The introduction of STPs resulted in a decreased release of these substances to recipients. Nevertheless the outgoing waters of STPs still contribute eutrophicating substances. Furthermore, various chemicals end up in the sewage and consequently gather in STPs which then act as point sources for pollutants that are not efficiently removed (Naturvårdsverket, 2010).

The comprehension that chemicals are released from STPs has been given increasing focus the last decades. Chemical analysis of STP effluent water and surface water of recipients shows that steroid hormones and pharmaceuticals still exit as active substances after treatment (Camacho-Muñoz *et al.*, 2010; Carballa *et al.*, 2004; Martìn *et al.*, 2012; Zorita *et al.*, 2009). The pharmaceuticals found in effluent water are those most extensively used, such as anti-inflammatory substances/pain killers, antibiotics, lipid regulators, beta blockers and synthetic hormones (Christen *et al.*, 2010). In general the main load of pharmaceuticals which enters the

waste water system come from excretion by users, whereas a smaller amount is discarded directly, for example in toilets (Kaplan, 2013). In addition production facilities have been shown to be major contributors at certain locations, resulting in particularly high concentrations in recipients (Larsson *et al.*, 2007; Phillips *et al.*, 2010). After usage, pharmaceuticals are excreted either as the mother compound or as metabolites. The conjugated metabolites are usually inactive but can be reactivated by microbes in STPs or in the environment (D'Ascenzo *et al.*, 2003).

The purpose of pharmaceuticals is to produce specific physiological responses in target organisms, mainly in humans but also in animals. Since vertebrates have similar endocrine systems, metabolic pathways and molecular targets, there is risk for unwanted effects in non target organisms exposed to pharmaceuticals in the environment (Christen *et al.*, 2010). The cases of intersex (oocytes in males) and vitellogenin (egg yolk precursor) induction in wild populations of fish living in rivers receiving STP effluents have been attributed to estrogenic contaminants and anti-androgens (Bjerregaard *et al.*, 2006; Blazer *et al.*, 2007; Jobling *et al.*, 1998). How severe these effects are on populations of wild fish is hard to know, but since the pharmaceuticals affect the physiology of the reproductive organ the ability to reproduce may be decreased (Kidd *et al.*, 2007). Furthermore estrogenic substances may alter the mating behavior rendering the fish less successful in breeding. In fact fish exposed to environmentally relevant levels of an anti-anxiety drug have shown increasing feeding rates and reduced sociability (Brodin *et al.*, 2013). Induction of genes involved in xenobiotic metabolism and oxidative stress in fish exposed to STP effluents also indicate that other adverse effects may arise (Albertsson *et al.*, 2009;

Cuklev *et al.*, 2012; Jönsson *et al.*, 2002; Samuelsson *et al.*, 2011; Sturve *et al.*, 2008). The realization that pharmaceuticals probably already affect wild biota in recipients of STP effluent has led to concern. What may be even more worrying is that drinking water has been found to contain pharmaceuticals (Kaplan, 2013). So far the risks for human health impacts due to pharmaceuticals in drinking water is considered very unlikely according to a report from The World Health Organization (WHO, 2012). However with the global increase of the human population and the increased industrialization the consumption of pharmaceuticals will likely increase, and with that also the concentration in STP effluents. In Sweden the sale of defined daily doses (DDD) of pharmaceuticals per 1000 inhabitants has increased from 1256 to 1717 from the year 2000 to 2012, corresponding to an increase of almost 27 %. During the same time period the economic value of sales increased from 21 to 36 billion SEK, which is an increase of 41% (Apotekens Service, 2013). The need for increased removal efficiency for pharmaceuticals in STPs has prompted a surge of research on additional treatment steps. Methods such as chemical oxidation processes by adding ozone, chlorine dioxide or hydrogen peroxide have shown great reduction of pharmaceuticals. Of these methods ozonation seems like the most promising alternative (Oulton *et al.*, 2010). However studies indicate that toxic intermediates are formed during oxidation treatments, resulting in induction of stress proteins, reduced survival or growth rates in test organisms (Cuklev *et al.*, 2012; Gagné *et al.*, 2007; Stalter *et al.*, 2010b). Other methods capable of increasing removal rates are filtration through nanomembranes and reverse osmosis. Yet another method is to use a membrane bioreactor which combines membrane filtration with biodegradation of pollutants. This method is considered more efficient than conventional activated sludge

processes but is not as efficient as oxidative methods (Oulton *et al.*, 2010). The seemingly safest method with high removal rates of pharmaceuticals is using activated carbon as a sorbent. By absorption of pollutants the carbon literally removes the substances without creating any new compounds. The downside is that activated carbon has to be renewed since it will be saturated.

The present study aimed to evaluate the additional treatment steps of ozonation and activated carbon. The study was carried out in Käppala STP, a plant that receives water from approximately 500 000 persons in the Stockholm area. Eighty % of the water comes from households and the rest from schools, hospitals, offices and industries. Every year around 55 million m^3 of water is treated. Ninety nine % of the organic material, at least 95 % of the phosphorus and 80 % of the nitrogen are removed during treatment. The first treatment step is mechanical removal of coarse materials and sand. The second step is presedimentation where particles precipitate and are collected in a biogas chamber. The third step is an activated sludge process where microorganisms biodegrade organic matter in both aerobic and anaerobic environments. In this step a lot of the nitrogen leaves as gas and phosphorus is incorporated in microbial biomass which becomes sludge. The fourth step is post sedimentation in a chamber where the sludge sediments before being partly pumped back to the activated sludge chamber, partly pumped to the biogas chamber. The fifth step is chemical treatment where iron sulfate is added to make the left over phosphorus precipitate. The sixth and final step is a sand filter where the remaining particles get stuck. The effluent water is released in the Stockholm archipelago at a depth of 45 meters. The left over sludge is considered of high quality and is used to

make soil or as fertilizer in agriculture. The produced biogas is used to fuel local busses. In addition heat is recycled and used for heating the STP and local residences (Käppalaförbundet, 2010).

In this project, small scale pilot equipment was used to treat the effluent with ozone and activated carbon. In order to evaluate removal of biologically active chemicals after the additional treatments we exposed rainbow trouts to differently treated water and measured gill EROD activity and a number of biomarker responses. The genes analyzed are involved in oxidative stress response, xenobiotic metabolism and the response to estrogen receptor activation. Below follows a short description of why oxidative stress is important for this study and the gene families analyzed.

Reactive oxygen species

Oxygen is critical for basic metabolism in aerobic organisms. At the same time it is highly reactive and unavoidably leads to the formation of reactive oxygen species (ROS). Another source of ROS is oxidation reactions which are important for oxidizing endogenous substances but also xenobiotics. Xenobiotics may also enhance the production of ROS by depleting or inhibiting antioxidants and interrupting the electron transport chain. Furthermore macrophages, neutrophils and monocytes use ROS in immune responses, creating a cytotoxic environment against pathogens (Kelly *et al.*, 1998). ROS can damage cells by oxidizing DNA, lipids and proteins. Healthy organisms are usually able to keep the levels of ROS low with a variety of defense mechanisms. However due to disease or extensive exposure to xenobiotics the defense mechanisms may be unable to control

the levels of ROS and the organism will suffer from oxidative stress (Hellou *et al.*, 2012).

Superoxide dismutase

Superoxide dismutases (SODs) catalyze the dismutation of the ROS superoxide anion ($^{\cdot}O_2^-$). SODs contain a transition metal group which is reduced by the $^{\cdot}O_2^-$ and re-oxidized by another $^{\cdot}O_2^-$ molecule, resulting in the production of oxygen (O_2) and hydrogen peroxide (H_2O_2). H_2O_2 may be a substrate for formation of hydroxyl radicals ($HO^{\cdot}$), but is efficiently metabolized by the enzyme catalase to yield water (Kelly *et al.*, 1998).

Glutathione S-transferase

Glutathione S-transferases (GSTs) catalyze the phase II conjugation of reduced glutathione to substances with an electrophilic center. The conjugation usually renders the substance less toxic and more water soluble thus increasing the excretion rate. Amongst these substances are the endogenous byproducts of lipid peroxidation which is propagated by the presence of ROS. Furthermore xenobiotics like pharmaceuticals can act as targets for conjugation (Leaver and George, 1998). Similarly to other antioxidant genes the GSTs are supposedly induced by oxidative stress. By starting a protein kinase signaling pathway, oxidative stress stimulates transcription factors to bind to the antioxidant response element (ARE) in respective promoter region of the genes, increasing the transcription (He *et al.*, 2013; Leaver *et al.*, 1997).

Metallothionein

Metallothionein (MT) is considered to fill two important roles. One is to detoxify nonessential and excess essential metals by binding to them, making them inactive. The other is to scavenge for free radicals, such as ROS and prevent oxidative stress (Carpené *et al.*, 2007). MT is inducible by many heavy metals. Following metal exposure the metal response element-binding transcription factor 1 (MTF-1) translocates into the cell nucleus binding and activating the metal response element (MRE) in the promoter region of the MT gene, thereby increasing the transcription. MT is also induced to a lesser extent by oxidative stress via the ARE promoter region (Haq *et al.*, 2003).

Heat shock proteins

Heat shock proteins (HSPs) are important for proper folding and refolding of damaged proteins. In addition HSPs interact with many signaling substances involved in the apoptosis pathway, acting as cytoprotective molecules. HSPs are induced by a variety of stressors, such as infection, inflammation, starvation, exposure to xenobiotics, hypoxia, temperature fluctuations etc. During non-stressful conditions HSPs bind to the heat shock factor (HSF), preventing it from binding to the heat shock element (HSE) promoter region. During stress events the amount of unfolded proteins increases. Since HSPs preferentially bind to unfolded proteins the HSF is released and able to trimerize. The trimeric state translocates into the nucleus and binds to HSE regulating HSP production (Padmini and Whitacre, 2010).

20β-hydroxysteroid dehydrogenase/Carbonyl reductase

20β-hydroxysteroid dehydrogenase (20β-HSD) plays an important role in final maturation of oocytes by producing maturation inducing hormone from endogenous steroids. The teleost 20β-HSD has high similarity to mammalian carbonyl reductase (henceforth called CBR) which also has a wide range of specificity for xenobiotics (Tanaka *et al.*, 2002). The suggested induction of CBR occurs by secondary messenger (cAMP) responsive elements to stimulate oocyte maturation but also by xenobiotic and/or oxidative response elements (Sreenivasulu *et al.*, 2012).

Vitellogenin and vitelline envelope proteins

Vitellogenin (VTG) is an egg yolk precursor produced in the liver of females during oocyte maturation (Mommsen and Walsh, 1988). The vitelline envelope proteins (VEPs) are structural and functional proteins surrounding and protecting the egg and embryo. The mRNAs for these proteins are usually induced by endogenous estrogens activating the estrogen receptor in females but can also be induced by xenoestrogens present in food or water. In males these proteins fill no purpose but can nonetheless be induced by exogenous estrogenic substances (Hyllner *et al.*, 2001).

Cytochrome p450

Cytochrome p450s (CYPs) are a superfamily of heme containing phase I enzymes. They are involved in the oxidation, reduction and peroxidation of many endogenous and exogenous substances. Therefore the CYPs are important for normal physiological functions such as biosynthesis of steroids, metabolism of fatty acids and prostaglandins as well as for detoxification of many chemicals. Most xenobiotics are rendered less toxic

and more hydrophilic by CYP-catalyzed reactions, facilitating the excretion or further metabolism by phase II conjugation enzymes (Buhler and Wang-Buhler, 1998). CYP1A1 has been shown to be induced by various polyaromatic hydrocarbons (PAHs), dioxins, polychlorinated biphenyls (PCBs), furans and some pesticides. The well accepted pathway for induction is believed to be through the aryl hydrocarbon receptor (AhR). As a ligand binds to the AhR it translocates from the cytosol to the nucleus where chaperone proteins also bind to the complex, finally binding to the xenobiotic response element (XRE) regulating gene expression. CYP1A3 has very similar amino acid sequence to CYP1A1 and has been shown to be induced by some of the same inducers. However the expression patterns are not identical. In some cases large differences in expressions are seen during single exposures, indicating differences in ligands and/or signaling pathways (Cao *et al.*, 2000). The induction of CYP3A45 is not well known but is believed to occur by endogenous steroids and exogenous compounds, much like the other mentioned CYPs (Lee and Buhler, 2003).

Material and methods

Fish, exposure and sampling

Juvenile rainbow trout (bought from Näs fiskodling AB, Sweden) were kept at the Evolutionary Biology Centre, Uppsala University. Tanks containing the fish were continuously provided with aerated tap water (7±0.5 °C). The fish were fed daily rations (2 mm pellets Inicio 917, Biomar, Brande, Denmark) corresponding to approximately 1% of their body mass. The experiments were approved by the local ethical committee.

On the sixth of March 2013, six days prior to the experiment 36 fish (48±9 g, mean body weight ± standard deviation) were moved to Käppala STP. The fish were randomly distributed into four 50 liter glass aquaria (biomass corresponding to 9 g/l) supplied with flow through aerated tap water (17 l/h by peristaltic pumps) and left to acclimate (14.98±1.28 °C). To avoid uneven feeding and accumulation of feces in the water the fish were not fed throughout the acclimation and exposure. On the eleventh of March 2013, after the six day acclimation period, three aquaria received differently treated water instead of tap water. One aquarium was left with tap water serving as control. Fish in the first exposure group were exposed to regular STP effluent water. The second group was exposed to effluent water filtered through granular activated carbon. The third group was exposed to effluent water treated with ozone (7 mg O_3/l). Temperature was logged every half minute. Oxygen saturation, pH and conductivity were measured every day. On the eleventh of March 2013, upon the fifth day of exposure, the fish were killed randomly by a blow to the head, thereafter weighed, length was

measured and the fish were sexed. Two gill arches from each fish were stored in ice cold Hepes-Cortland (HC) buffer for gill EROD assay the same day. The remaining gills and a piece of liver were excised and frozen in liquid nitrogen and brought back to Uppsala for gene expression analysis (qPCR).

EROD

EROD stands for 7-ethoxyresorufin O-deethylase. The process of O-deethylation of 7-ethoxyresorufin into resorufin is catalyzed by enzymes from the CYP1 family. After excitation resorufin gives off fluorescence and thus the resorufin concentration can be determined. By immersing gill filaments in a solution containing 7-ethoxyresorufin it is possible to determine the formation of resorufin attributed to the CYP1s present in the gill.

In short, the gill arches kept in ice cold HC buffer were brought back to Uppsala University for analysis the same day as the exposure ended. Twenty gill filament tips (2 mm long) were cut off from each fish and transferred with Pasteur pipette into two wells in a 12-well plate containing HC buffer, giving 10 tips in each well. The HC buffer was replaced with a reaction buffer containing 7-ethoxyresorufin. After ten minutes the buffer was renewed. Reaction buffer was then sampled at two time points (around 30 and 50 minutes) and put in 96-well plates. Resorufin standards (0.1-250 nM) were prepared and put in 96-well plates. The fluorescence was measured at 590 nm after excitation at 544 nm in a multi-well plate reader Victor3 (Perkin Elmer, Waltham, MA 02451, USA). The readings from the

standards were used to make a curve where the linear part was used to get the equation to calculate the amount of resorufin formed in the gill samples (Jönsson *et al.*, 2002).

RNA extraction, cDNA synthesis and quantitative PCR

Gene expression was analyzed using quantitative polymerase chain reaction (qPCR), which is a fluorescence-based method to measure gene amplification. mRNA from tissues of sampled fish is used to make complementary DNA. The cDNA is used in a PCR in combination with primers and a reporter dye which gives off fluorescence, making it possible to measure the amplification of specific DNA sequences. By comparing amplification of DNA in exposed fish to control fish it is possible to get a relative gene expression value.

To extract total RNA, gill and liver samples were thawed and homogenized with a Bullet Blender Storm 24 and 0.5 mm zirconium oxide beads (Next Advance Inc, Averill Park NY 12018, USA). Total RNA was extracted using Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories Inc, Hercules CA 94547, USA). In short, 200 µl PureZOL™ RNA isolation reagent and the beads (1:1 ratio sample/beads) were added to the samples in snap lock reaction tubes. Following a two minute run in the Bullet Blender another 800 µl PureZOL™ was added. The initial amount of PureZOL™ was added to facilitate the disruption and homogenization of cells and tissue. Thereafter a series of centrifugation steps were made, initially to get rid of insoluble debris. After addition of chloroform another centrifugation was made, where the supernatant divides into three phases.

The upper phase containing RNA was saved in an RNA-binding column while the other phases containing DNA and proteins were discarded. Later several centrifugation steps followed where washing solution was added between each step. DNase 1 was added to be certain that the samples were free from genomic DNA. Finally RNA elution solution was added to the binding column membrane followed by a short centrifugation, which eluted the total RNA.

Total RNA purity and quantity were controlled spectrophotometrically with Nanodrop 2000c (NanoDrop Technologies, Wilmington DE 19810, USA). Using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc, Hercules CA 94547, USA) the RNA was reversely transcribed into cDNA. In short, after confirming RNA purity and quantity the samples were diluted to contain the same amount of RNA (1000 ng). cDNAs were synthesized by adding iScript reverse transcriptase and 5x iScript reaction mix (containing oligo dT and random hexamer primers) to the samples and incubating in an iCycler (Bio-Rad Laboratories Inc, Hercules CA 94547, USA) for 5 minutes at 25 °C, 30 minutes at 42 °C and 5 minutes at 85 °C.

Real-time qPCR was performed for 17 genes with gene specific primers (synthesized by Sigma-Aldrich) (table 1). Samples (20 µl) consisting of cDNA (made from 1000 ng RNA), forward and reverse primer (5 pmol each) and iQSYBR Green Supermix (Bio-Rad Laboratories Inc, Hercules CA 94547, USA) were run in duplicates in a Rotor-Gene 6000 real time DNA amplification system (Qiagen, Hilden, Germany) for 95 °C (10 minutes) followed by 40 cycles of 95 °C (15 seconds) and 62 °C (60 seconds). By analyzing melt curves it was possible to confirm that only a

single product had been produced. Each primer pair efficiency (E) was calculated in the LinRegPCR software (Ruijter *et al.*, 2009). The efficiency value theoretically ranges between 1 and 2 denoting at what rate amplification occurs. If E=2 every PCR cycle will result in duplication of the sequence. The efficiency value was used to calculate relative gene expression using the $E^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The equation:

$$E^{-\Delta\Delta Ct} = E^{(A\,\mathrm{Ct}_{\text{gene of intrest}} - \mathrm{Ct}_{\text{reference gene}}) - (B\,\mathrm{Ct}_{\text{gene of intrest}} - \mathrm{Ct}_{\text{reference gene}})}$$

Where *A* represents exposed fish and *B* control fish. The method gives a fold change value compared to the control with adjustment to a reference gene. The equation relies on Ct (cycle threshold) values which are the numbers of polymerase chain reactions it takes for the amplification curve to reach a manually set threshold in the exponential phase.

Table 1. Genes and primer nucleotide sequences used for quantitative PCR.

Gene	Sequence (5'-3')	Genebank accession number.
B-actin	F-CCTCGGTATGGAGTCTTGC R-CTTACGGATGTCCACGTCACA	NM_001124235.1
CBR A	F-CAGGAAGTTTACCTCATCTCACAGAC R-AAAAGCTAAGATATGTCAACAACCCTAT	NM_001124596.1
CBR B	F-CATTGCAAGGGTTGTGGAGAT R-CATTACAATGAAAGTACTCCAGAGTGTTATTTAT	NM_001124255.1
CYP1A1	F-GGAAACTAGATGAGAACGCCAACA R-GTACACAACAGCCCATGACAG	AAB69383.1
CYP1A3	F-GAAACTAGATGAGAACGCCAACG R-CTGATGGTGTCAAAACCTGCC	NM_001124754.1
CYP3A45	F-AGACTGCGTTGTCCTGGTTC R-ATGTACGTGTACGGATCAATAGG	AF267126.1
EF-α	F-GCAGGTACTACGTCACCATCAT R-CACAATCAGCCTGAGATGTACC	NM_001124339.1
GST A	F-CAGAGGACAGCTCCCTGCTT R-CTGAACCGGCTCTCCAGGTA	NM_001160559.1
HSP70	F-CTGCAACTAACTCCAAACCATCAA R-GTTGGCGATGATCTCCACTTT	NM_001124228.1
HSP90	F-GAGGACAAGACAAAGTTTGAGAACC R-GACACGGTCACCTTCTCTACTTTCT	NM_001124231.1
MT	F-ATGGATCCTTGTGAATGCTCTAAA R-GGGCAGCAACTTTTCTTACAACT	M18104.1
SOD Cu/Zn (1)	F-CCAGTAAACAGGACACACTCCCTTA R-GGCCGTAGCTACAGTGGTCTTC	NM_001124329.1
SOD Cu/Zn (2)	F-GGTCCTGTGAAGCTGATTGG R-CTCATACAGCCGTTGGTGTTGT	NM_001124329.1
VTG	F-ATCGAGAGGCTGGAGTTTGA R-TCCTCCTCTTCGTTCATGGT	X92804.1
Zp1 (VEP)	F-CAGTGACTGTCCAGTGTACCAA R-GGCTGATGGAATCTAGCTCAA	NM_001124273.1
Zp2 (VEP)	F-CCACCCTATTGGCACAACTTC R-CTCCGTCATGACAGTTCCACATT	NM_001124600.1
Zp3 (VEP)	F-GCCAGCTAAGGATGACTACCAACT R-GATCATCGCGTCATTTGTCC	NM_001124274.1

F=forward primer, R=reverse primer

Primer design

In order do design primers rainbow trout gene sequences were acquired from GenBank: http://www.ncbi.nlm.nih.gov/gene except the primers for the CYP genes which are previously described in Jönsson *et al.* (2010). To avoid amplification of genomic DNA, primers should be located in two different exons or over the exon-exon border. No such information about rainbow trout exon borders existed in GenBank. However the exon borders are known for zebra fish *(Danio rerio).* Due to the putative conservation of genes between rainbow trout and zebra fish an assumption was made that the exon borders were placed in similar locations in both species. Therefore the protein sequence (translation product) for the different rainbow trout genes were Blasted (BlastP in Ensembl: http://www.ensembl.org/Multi/blastview) against zebra fish proteins. Subsequently the DNA sequence in zebra fish coding for the particular protein was aligned (in CLC Sequence Viewer v 5.0, CLC bio) to the corresponding sequence in rainbow trout. It was then possible to place the primers in assumingly different exons or over an exon-exon border. Melting temperatures of the primer pairs were checked to not differ more than 3 °C, but one exception was made for the primers for Zp3. The primer pairs were checked (in Amplify 3 v 3.1.4, Bill Engels, University of Wisconsin) to have a low risk for primer dimerization and lack secondary binding sites and to avoid mispriming they were checked for low binding capability in the 3' end. For each gene three primer pairs were designed and purchased (from Sigma-Aldrich). To evaluate the most fitting primer pair for each gene a qPCR run was performed in duplicates with cDNA from pooled rainbow trout samples. From inspection of the melt curve some primer pairs were excluded due to low or high melting temperature, indication of secondary structures or large differences in the duplicates. To be certain that

the wanted products had been amplified the product of the qPCR run were stained with ethidium bromide and run in 1 % agarose gel (1x TAE running buffer) by electrophoresis (RunOne™ system Embi Tec, San Diego, CA 92126, USA) against a 1 Kb plus DNA ladder. After confirmation that a single DNA product of expected size was present the gel slices with the product were excised and the DNA extracted using MinElute® Gel Extraction Kit (Qiagen, Hilden, Germany). In short, the excised gel slice was dissolved in buffers and transferred to a DNA binding column. After three centrifugation steps with different buffers added in between each step the DNA is extracted from the gel, purified and ready to be eluted. The eluted DNA samples were sent with forward and reverse primer separately to Uppsala Genome Center for sequencing. The sequences were analyzed in Blast (BlastN in http://blast.ncbi.nlm.nih.gov/Blast.cgi) and confirmed to be correct.

Statistics

The EROD activities and relative gene expressions between fish in different exposure groups were statistically tested by one-way ANOVAs followed by Tukey's post hoc test in GraphPad Prism® v 5.01 (GraphPad Software Inc., CA, USA). If variances differed significantly between groups log-transformed values were used. Values are presented as mean fold change compared to control with standard deviation (SD). One fish from the control group was excluded from the calculations since cDNA quantity was insufficient which led to unacceptably high Ct values.

Results

Temperature, pH and oxygen saturation were fairly similar and stable in every treatment. Conductivity was substantially lower in the tap water control compared to the differently treated effluent water (table 2).

Table 2. Mean (±SD) for water parameters in different exposure groups.

Exposure group:	Control	Effluent	Activated carbon	Ozone
Temperature (°C)	13.2±0.5	12.9±0.1	13.6±0.4	14.1±0.5
pH	7.8±0.2	7.3±0.1	7.6±0.1	7.8±0.1
Conductivity (mS)	228±7	710±13	722±5	716±7
Oxygen saturation (%)	92.9±0.7	91.7±0.5	92.4±0.8	91.8±0.5

The gill EROD activity was increased 4.5-fold in fish exposed to effluent water compared to the control fish. The EROD activity in gills was significantly lower in fish exposed to both activated carbon filtered and ozonated water compared with the activity in fish exposed to effluent water (figure 1). The activated carbon treatment resulted in very low EROD activity, even significantly lower than the activity in the tap water control fish.

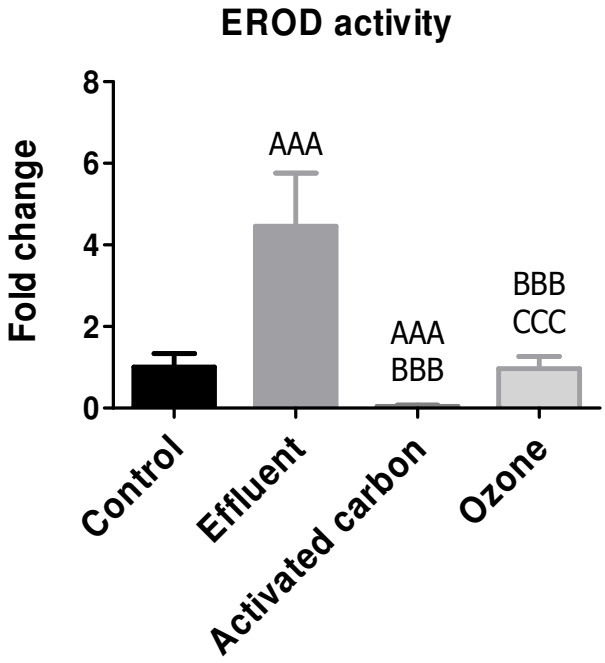

Figure 1. EROD activity (mean + SD) in gills of rainbow trout (n=9/group) exposed for five days to differently treated sewage treatment plant water. Statistical significance was tested by one-way ANOVA followed by Tukey's post hoc test. Significance compared to control is denoted as A, to effluent as B and to activated carbon as C. The number of letters indicate the significance level (X=p<0.05, XX=p<0.01 and XXX=p<0.001).

The CYP1A genes were the most influenced by the different exposures. CYP1A1 and CYP1A3 mRNAs were induced 3.5- and 3.1-fold respectively, in gills of fish exposed to effluent water compared to control fish. Likewise both genes were down regulated in water filtered through activated carbon compared to both control and effluent treatments. Furthermore exposure to ozone treated water resulted in significantly lower expression of both genes compared to effluent but significantly higher compared to activated carbon (figure 2). The expression pattern of the CYP genes in liver samples showed similar patterns, but the highest induction of both genes was found in the fish exposed to ozonated water. Two- and 2.3-fold increases compared to the control were found for CYP1A1 and CYP1A3, respectively (figure 2).

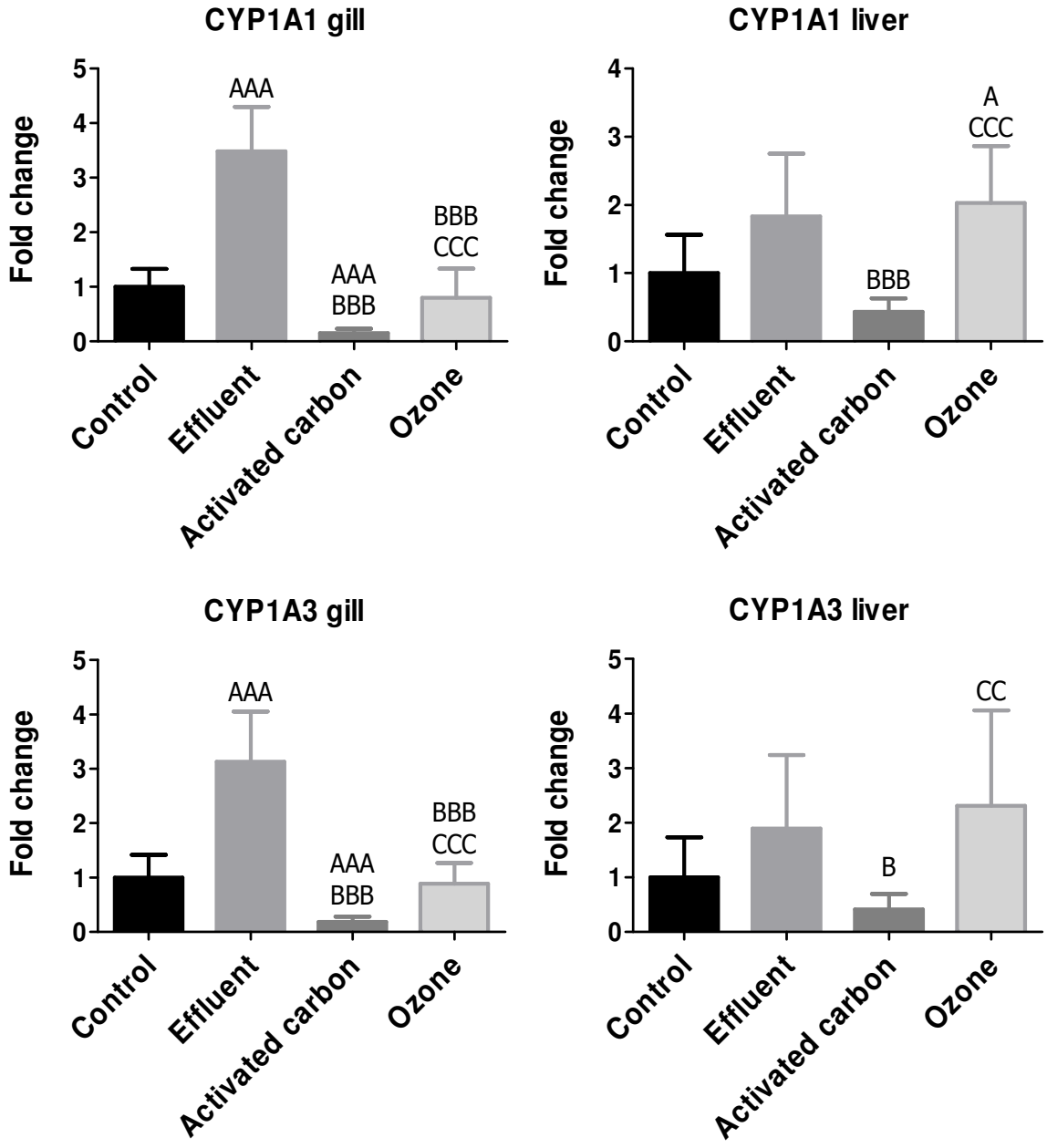

Figure 2. Relative mRNA expression (mean + SD) of CYP1A genes in gills and liver of rainbow trout (Control n=8, Effluent n=9, Activated carbon n=9 and Ozone n=9) exposed for five days to differently treated sewage treatment plant water. Calculations were made with EF-α as reference gene and tap water controls as calibrators. Statistical significance was tested by one-way ANOVA followed by Tukey's post hoc test. Significance compared to control is denoted as A, to effluent as B and to activated carbon as C. The number of letters indicate the significance level (X=$p<0.05$, XX=$p<0.01$ and XXX=$p<0.001$).

The CBR A and B isoforms showed similar patterns but there were differences between gill and liver. In gills the largest difference compared to control fish was seen in fish exposed to ozonated water. A 2.6- and 2.2-fold higher mean could be seen for the A and B forms respectively. Notably, however only the value for CBR A was significantly different from the control value (figure 3). In liver samples CBR mRNA levels varied a lot and there were no significant differences between the groups. For both CBR A and CBR B, the numerically lowest transcript level was found in the fish exposed to activated carbon filtered water.

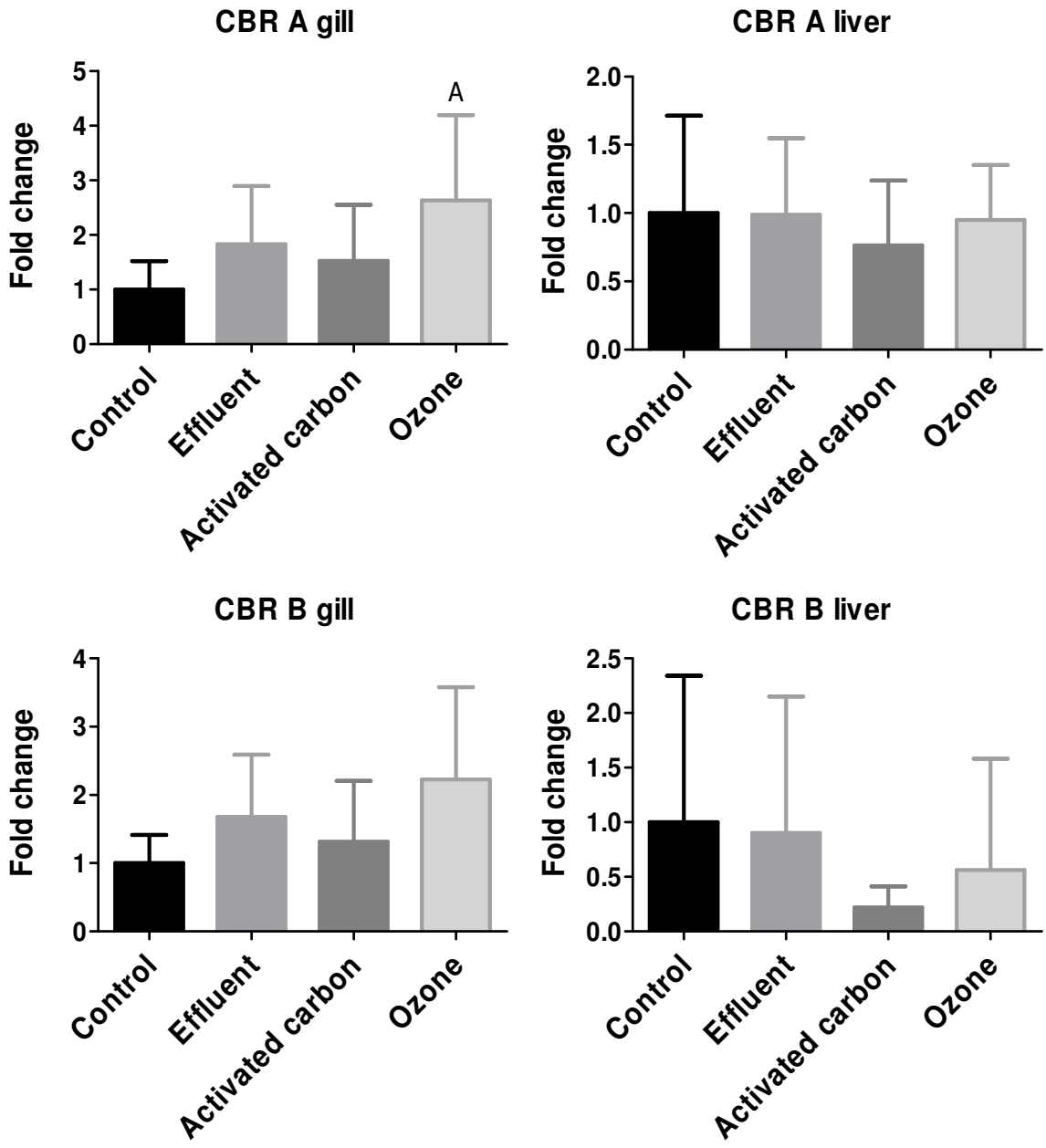

Figure 3. Relative mRNA expression (mean + SD) of carbonyl reductase (CBR) gene isoforms A and B in gills and liver of rainbow trout (Control n=8, Effluent n=9, Activated carbon n=9 and Ozone n=9) exposed for five days to differently treated sewage treatment plant water. Calculations were made with EF-α as reference gene and tap water controls as calibrators. Statistical significance was tested by one-way ANOVA followed by Tukey's post hoc test. Significance compared to control is denoted as A (A=$p<0.05$).

GST A was fairly unaffected by the different treatments compared to control, but fish exposed to water filtered through activated carbon showed significantly lower expression in gills than those exposed to effluent (figure 4).

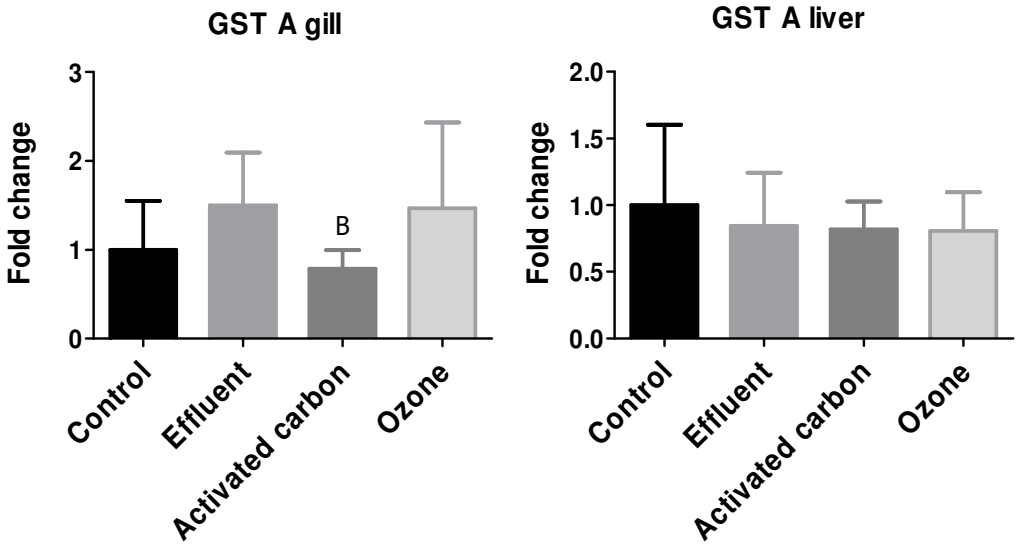

Figure 4. Relative mRNA expression (mean + SD) of the glutathione S-transferase A (GST A) gene in gills and liver of rainbow trout (Control n=8, Effluent n=9, Activated carbon n=9 and Ozone n=9) exposed for five days to differently treated sewage treatment plant water. Calculations were made with EF-α as reference gene and tap water controls as calibrators. Statistical significance was tested by one-way ANOVA followed by Tukey's post hoc test. Significance compared to Effluent is denoted as B (B=$p<0.05$).

A non-significant 8.7 fold higher value of HSP70 in fishes exposed to ozonated water compared with the tap water control could be seen in gills (figure 5A). This effect was largely due to four fishes having high values and hence there was a large standard deviation. In liver, HSP70 was fairly similar in all exposures (figure 5A). MT mRNA was somewhat higher in fish gills exposed to effluent and ozonated water compared to control and activated carbon but the differences were not significant. In liver, although not significant all exposed groups showed lower values compared to control (figure 5B).

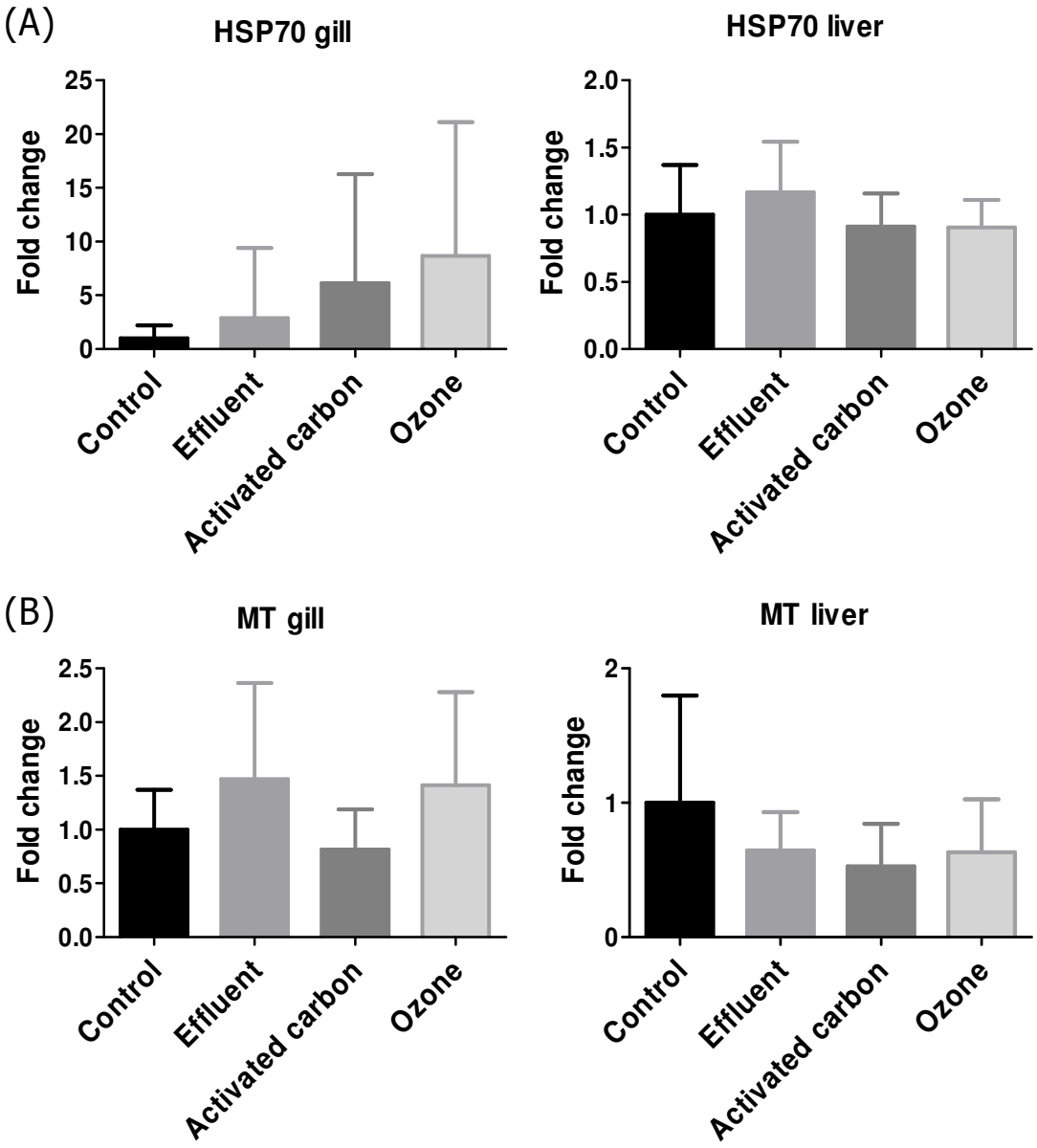

Figure 5. Relative mRNA expression (mean + SD) of (A) heat shock protein 70 (HSP70) and (B) metallothionein (MT) in gills and liver of rainbow trout (Control n=8, Effluent n=9, Activated carbon n=9 and Ozone n=9) exposed for five days to differently treated sewage treatment plant water. Calculations were made with EF-α as reference gene and tap water controls as calibrators. Statistical significance was tested by one-way ANOVA followed by Tukey's post hoc test.

The transcription levels of estrogen responsive genes were mainly lower in most exposure groups compared to control. However the only significantly down regulated gene compared to the control was the vitelline envelope protein Zp2 in fish exposed to ozonated water (figure 6).

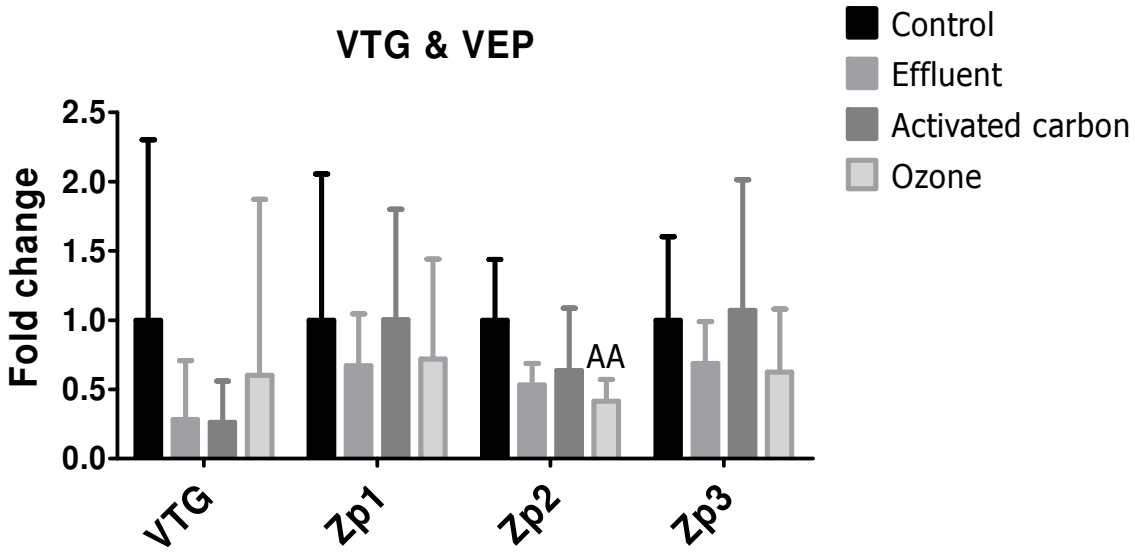

Figure 6. Relative mRNA expression (mean + SD) of the estrogen dependent genes vitellogenin (VTG) and vitelline envelope proteins (VEPs) zona pellucida 1, 2 and 3 (Zp1, Zp2 and Zp3) in liver of rainbow trout (Control n=8, Effluent n=9, Activated carbon n=9 and Ozone n=9) exposed for five days to differently treated sewage treatment plant water. Calculations were made with EF-α as reference gene and tap water controls as calibrators. Statistical significance was tested by one-way ANOVA followed by Tukey's post hoc test. Significance compared to Control is denoted as A (AA=$p<0.01$).

CYP3A45, SOD Cu/Zn and HSP90 showed little variation between different treatments and were comparable to the control group in both gills and liver (data not shown).

Discussion

The most obvious effects on gene transcription were the induction of CYP1A1 and CYP1A3 mRNA in rainbow trout gills exposed to STP effluent water compared with the tap water control and the much lower expression of these genes after activated carbon filtration and ozonation of the effluent water. These effects are supported by the similar pattern of the gill EROD activity. These results indicate that AhR agonists probably are present in the effluent water but are removed to a great extent through additional treatment of the effluent water. Somewhat surprising is the CYP induction in liver of fish exposed to ozonated water, since the gill samples showed a lower expression compared to effluent water. Other studies suggest that readily metabolized compounds such as PAHs may undergo first-pass metabolism in the gills and therefore never influence the gene expression in liver. In contrast more persistent compounds such as PCB 126 may have the ability to reach the liver (Brammell *et al.*, 2010; Jönsson *et al.*, 2006). In a 15 k rainbow trout hepatic microarray an induction of CYP1A genes was seen in effluent water, an effect that was extinguished in the ozone and activated carbon treatment (Cuklev *et al.*, 2012).

CBR A and B have previously been shown to be induced in liver of rainbow trout exposed to STP effluents, the model AhR agonist β-naphthoflavone and the prooxidant herbicide paraquat. The same study showed that CBR expression in fish exposed to STP effluent treated with ozone was similar to that of the control group (Albertsson *et al.*, 2009). The present study could not support that CBR was induced in fish exposed to STP effluent, although a slightly higher expression was seen. In contrast the fish exposed to

ozonated effluent showed a significantly increased value of CBR A in gills compared to control. This may indicate that ozonation is not able to reduce the xenobiotic concentration or that it results in formation of new chemicals causing oxidative stress. Even though not significant the higher expression of GST A, HSP70 and MT in gills of fish exposed to ozonated effluent indicates the same. The previously mentioned 15 k rainbow trout hepatic gene expression microarray also showed increased expression of HSP70, HSP90 and GSTs in ozonated water (Cuklev *et al.*, 2012).

In the present study the estrogen responsive genes were generally not differently expressed between exposure groups. The relative expression of these genes was erratic. Typically a few fish per group, especially in the control showed high values compared to the rest, making the standard deviation large. In other studies these estrogen responsive genes are usually induced in effluent water and down regulated in groups exposed to additionally treated water (Cuklev *et al.*, 2012; Stalter *et al.*, 2010b). The difference seen in the present study made in the Käppala STP compared to other studies is hard to interpret since similar treatments are used in all the STPs studied. One possible reason for our result may be that sewage effluents do not only contain estrogenic substances but also anti-estrogenic substances. The anti-estrogenic effects are usually masked by the stronger estrogenic effect (Conroy *et al.*, 2007; Stalter *et al.*, 2011). If in this case the estrogenic substances were already at low concentrations in effluent water the anti-estrogenic substances might have had a detectable effect by down regulating the estrogen responsive genes.

The qPCR method used in this study is highly sensitive for detecting changes in gene regulation. Then again this sensitivity only applies for the analyzed genes which in this study are involved in xenobiotic metabolism, oxidative stress and the response to estrogen exposure. The genes were chosen because they have previously been shown to be influenced in fish exposed to sewage effluent. They just make up a tiny part of a vast set of genes that are possibly influenced. There are more exploratory methods capable of detecting more general responses in exposed organisms. For instance using the previously mentioned 15 k gene expression microarray permits analysis of a large set of genes and determination of which genes that are the most different between treatments. The method gives a chance of detecting changes in gene expression that may go unnoticed if only a few genes are analyzed, but on the other hand microarrays are not as sensitive as qPCR in detecting small changes in gene expression (Cuklev *et al.*, 2012). Another exploratory method is to study the metabolome. In a recent study blood plasma of rainbow trout exposed to differently treated sewage water was analyzed for changes in metabolic fluxes (Samuelsson *et al.*, 2011). Metabolites will always be present in a living organism, but the metabolic profile will differ depending on the condition of the organism. The authors report that fish exposed to activated carbon filtered water had a similar metabolomic profile as control fish exposed to tap water. However the fish exposed to ozonated water (15 mg/l) showed diverging metabolomic profiles. For instance these fish had an increase in blood glucose which is a suggested response to stressors (Samuelsson *et al.*, 2011). Metabolomics may be excellent for finding differences between treatment groups but the data collected is vast and can be very hard to interpret. Besides tests in fish there are also ecotoxicological studies where organisms from different

trophic levels have been exposed to sewage effluent including additionally treated water. One study performed on a bacterium, a micro alga, a macro alga, a crustacean and embryos of a fish showed decreased negative effects of ozonated water compared to regular effluent (Lundström *et al.*, 2010a). Another study made on copepods also reports decreased adverse effects by ozonating the sewage effluent (Lundström *et al.*, 2010b). Other studies show that ozonation may in fact increase toxicity compared to effects caused by effluent or control water. Effects such as inhibition of growth in aquatic plants, increased mortality and emergence time of insect larvae, decreased biomass and number of aquatic worms and decreased survival of daphnids have been reported (Magdeburg *et al.*, 2012; Stalter *et al.*, 2010a). Furthermore a study on early life stages of rainbow trout showed reduced growth and increased mortality of fish exposed to ozonated water compared to effluent water (Stalter *et al.*, 2010b). The negative effects of ozonation mentioned in the three latter studies were all reduced by sand filtration post ozonation, indicating that an additional step after ozonation may be able to reduce the putative toxic byproducts.

In the present study no chemical analysis of the water was made, and consequently no conclusions can be drawn regarding what substance caused the induction or reduction in gene expression. In a number of the studies cited they have measured pharmaceuticals in the differently treated water and seen a great reduction by ozonation and activated carbon filtration. Even then it is hard to make any connection between reduced amounts of pharmaceuticals and biological responses since there are other chemicals present in the water which are not measured.

Conclusion

The aim of this study was to evaluate whether the additional treatments steps ozonation and activated carbon were able to reduce the amount of biologically active substances appearing in STP effluent water. The most solid evidence that these methods are in fact able to reduce the amount of chemicals was the reduction of EROD activity, CYP1A1 and CYP1A3 mRNA expression in fish following activated carbon filtration and ozonation compared to the effluent. The liver induction of CYPs in fish exposed to ozonated water may be due to fairly persistent AhR agonists. Although not significant in most cases the fish exposed to activated carbon treated water showed a slightly lower expression of most genes compared to fish exposed to effluent water, indicating the efficiency and relative safety of activated carbon. Regarding the genes involved in stress responses no solid conclusion can be made from the results. Nonetheless the expression of these genes was somewhat higher in the fish exposed to ozonated water. The expression of the estrogen responsive genes showed almost no difference between exposure groups, the most obvious conclusion would be that estrogenic contaminants already are at low concentrations not influencing these genes, but the possible antagonistic effects of anti-estrogens can not be excluded.

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