
# The Toxicological Evaluation of Sewage Effluents and Pharmaceuticals with the use of Zebrafish as a Model Organism

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The present thesis is a partial fulfilment of the requirements for a Master of Science Degree in Veterinary Medicine for International Students at the Swedish University of Agricultural Sciences (SLU), in the field of Aquatic Toxicology

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**To God and my family**

# Contents

## Abstract

The presence of pharmaceuticals in the environment has been an issue of increasing concern and sewage treatment plants have been identified as the principal sources of pharmaceuticals and endocrine disrupting chemicals in the aquatic environment.
In this study, zebrafish juveniles and adults were exposed to sewage effluents that had undergone treatment processes (A1-A7) at Hammarby Sjostad's sewage treatment plant in order to evaluate the effectiveness of the treatment processes. This was conducted with the aid of the fish embryotoxicity test (FET), reproduction test and the fish sexual development test (FSDT). A reduction in the spawning ability and fecundity (number of eggs produced by the females) was observed in the reproduction test in sewage effluent A4 (Biofilter). The zebrafish exposed to sewage effluent A2 (after sedimentation treatment) had a higher number of successful spawnings than the controls, while the fish exposed to effluents A3 (Outlet L1) and A5 (ozone) exhibited a decrease in spawning ability. The induction of vitellogenin was detected in male zebrafish exposed to A2 (after sedimentation treatment), A3 (Outlet L1) and A4 (Biofilter) treatment processes in the fish sexual development test (FSDT). In the FET conducted on the offsprings of the adult zebrafish exposed to the various sewage effluents, no effect was observed. In addition, eight pharmaceuticals from different therapeutic classes viz Clozapine (anti psychotic), Atenolol (β-blocker), Cimetidine (anti histamine), Fluoxetine (anti depressant), Loperamide (anti diarrhoeal), Verapamil (Calcium channel blocker), Bezafibrate (lipid lowering agent) and Cyclophosphamide (anti neoplastic) were evaluated for their potential toxicity to zebrafish embryos with the aid of the fish embryotoxicity test. The zebrafish embryos were exposed to five different concentrations of the pharmaceuticals in 96 well plates. The exposure concentrations were 1µg/L, 10µg/L, 100µg/L, 1mg/L and 10mg/L. Dimethyl sulphoxide (DMSO) and standardized fish water were used as controls. A concentration of 0.1% DMSO was maintained in the test solutions. A mixture of musk ketone and phenylthiourea was used as positive control. The endpoints monitored included hatching time, heart rate, tail extension, circulation, coagulation, spinal deformation, death and oedema. A decrease in the heart rate was observed in the zebrafish embryos exposed to the highest concentration of clozapine (10 mg/L) and abnormalities were observed in the embryos exposed to all the concentrations of clozapine.
The results of this investigation indicate that the FET could be improved upon in order to render it more sensitive for the toxicity testing of substances. Also, ozonation appears to be an effective treatment technique in sewage treatment plants for the reduction of sewage effluents and pharmaceuticals.



Keywords: Zebrafish, *Danio rerio*, pharmaceuticals, reproduction test, fish sexual development test, fish embryo toxicity test, vitellogenin

# Introduction

## Chemical Pollutants in the Environment

Pharmaceuticals and endocrine disrupting chemicals are chemical pollutants in the environment. The presence of pharmaceuticals in the aquatic environment has been an issue of increasing concern (Richardson and Bowron, 1985; Halling- Sorenson et al., 1998) since their occurrence in the aquatic environment was first reported in the mid-1970s (Hignite and Azarnoff, 1977). This has been attributed to their growing and unrestricted use (Dietrich et al., 2002). Chemical analysis methods such as liquid chromatography -tandem mass spectrometry have been developed to facilitate the detection of more polar pharmaceutically active compounds (Ternes et al., 1998; Ternes et al., 2001; Kolpin et al., 2002; Kummerer, 2004).

The main route of entry of pharmaceuticals into the environment has been identified as effluent from sewage treatment plants and the disposal of unused drugs down the drain or with household garbage (Jones et al., 2001). A large number of pharmaceuticals are partially eliminated during treatment in sewage treatment plants and this has resulted in their occurrence being detected in waste water treatment works (WTW) effluents, lakes, rivers, and rarely in groundwater (Hirsch et al.,1996; Hirsch et al .,1998; Ollers et al .,2001; Stackelberg et al.,2004). Pharmaceuticals have been mainly detected in the environment in proximity to sewage treatment plants, in waste water and in surface water in most countries (Halling-Sorensen et al., 1998; Kolpin et al., 2002; Gross et al., 2004). Several environmental analyses have been conducted in various countries and these monitoring studies clearly indicate that drug residues in treated waste water and surface water are very common (e.g. Halling- Sorensen et al., 1998; Daughton and Ternes, 1999; Kummerer, 2004).

The concentrations of pharmaceuticals detected in the environment are in the ng/L - µg/L range (Daughton and Ternes, 1999). Roberts and Thomas (2005) reported the occurrence of some pharmaceuticals in the Tyne estuary in the U.K. in the range of 4 -2370 ng/L.
The nonsteroidal anti inflammatory drug ( NSAID ), ibuprofen, and its metabolites have been observed to occur in STP effluents (Ternes,1998; Boyd et al., 2003; Weigel et al., 2004) in surface water reaching 1 µg/L (Kolpin et al ., 2002) and in seawater (Thomas and Hilton ,2004 ;Weigel et al., 2004).
Phenazone, propiphenazone and clofibric acid have been detected in samples of potable water in Germany (Heberer and Stan, 1997; Reddersen et al., 2002).

Most pharmaceuticals are capable of retaining their chemical structure in order to exert therapeutic effect and this characteristic, coupled with their continuous release into the environment may enable them to persist in the environment for extended periods of time (Ternes, 2000). For many drugs, metabolism occurs in the body following administration by the various routes and the drugs are reduced to inactive forms. After metabolism, some metabolites can remain physiologically

active or they could revert back to the active parent form (Hirsch et al.,1999). Other drugs applied topically, excreted or partially absorbed, have the propensity of leaving the body in their active states. Consequently, an array of pharmaceuticals, metabolites and their conjugates are being discharged into the effluent system (Ternes et al., 2001;Miao et al.,2002; Heberer, 2002).

Aquatic organisms have been identified as paramount targets of pharmaceuticals since they are continually exposed via wastewater residues throughout their entire life span (Fent et al., 2006). Apart from the detection of diclofenac in the prey of vultures, the bioaccumulation potential of pharmaceuticals in biota or food webs has not been reported (Oaks et al., 2004). Mimeault et al., (2005) noted that the concentrations of pharmaceuticals in fish can attain considerably higher concentrations in plasma compared to ambient water. Brooks et al.,(2005) remarked that fluoxetine and setraline, selective serotonin reuptake inhibitors (SSRIs), and their metabolites namely norfluoxetine and desmethylsertraline have been detected in fish. Based on the exposure concentrations, the bioconcentration factors of diclofenac (a non steroidal anti inflammatory drug) were observed to be 5 – 1000 in the kidney and 10–2700 in the liver of fish (Schwaiger et al., 2004).

Pharmaceuticals have been known to exert various effects on the aquatic system. Fluoxetine, a serotonin re-uptake inhibitor has been identified as the most acutely toxic human pharmaceutical with acute toxicity ranging from EC50 (48h, alga) = 0.024 mg/L (Brooks et al., 2003) to LC50 (48h) = 2 mg/L (Kummerer, 2004). Studies have indicated that selective serotonin reuptake inhibitors (SSRIs) induced spawning in zebra mussel (*Dreissena polymorpha*) and parturition in fingernail clam (*Sphaerium corneum*) (Fong et al., 2003). The anticonvulsants, diazepam and carbamazepine, are potentially toxic to aquatic organisms since most of the acute toxicity data recorded for them are below 100mg/L (Fent et al., 2006). The induction of strong bradycardia, accumulation of blood in the heart and arrhythmias were observed in 3 day old zebrafish embryos exposed to 10μM of astemizole (an H1 receptor agonist) for 1hr (Langheinrich et al., 2003). Mianserin (a second generation tricyclic antidepressant) induced alterations in gene expression in zebrafish after short term (2 days) and prolonged water exposure (14 days) at 250 μg/L (van der Ven et al., 2006).

Endocrine disrupting chemicals (EDCs) are substances that are capable of interfering with the hormonal systems of animals (Quinn et al., 2004).
They are capable of disrupting the reproductive systems of a wide range of species and this effect has been associated with untoward effects on the reproductive capabilities of wild life species (Guillette and Gunderson 2001; Kime 1998; Tyler et al., 1998; Van Der Kraak 1998). The effluents from municipal sewage treatment plants have been demonstrated to contain a complex mixture of EDCs such as xenoestrogens (e.g. alkylphenol polyethoxylates) and synthetic and natural hormones (e.g.17α- ethinylestradiol and 17β-estradiol) (Desbrow et al., 1998). Xenoestrogens act by mimicking or interfering with the endogenous hormones of animals by binding to the estrogen receptor and producing a biological response (Jobling et al., 1996; Hemmer et al., 2001). The presence of xenoestrogens has

been detected in concentrations up to tens of parts per billion, sufficient to elicit endocrine disruption in animals (Ahel et al., 1994; Ternes et al., 1999).

Ethinylestradiol (EE2) is a pharmaceutical that has been reputed to be a powerful endocrine modulator and its presence has been detected in the aquatic environment at biologically active levels (Nash et al., 2004).
EE2 has been detected in sewage effluent discharges and surface waters at concentrations between 0.5 and 7ng/L (Desbrow et al., 1998; Larsson et al., 1999; Ternes et al., 1999). According to a study conducted by Routledge et al., (1998), the degradation of synthetic estrogens (17 α- ethinylestradiol, diethylstilbestrol) and progestogens by microbial activity culminates in the release of their deconjugated biologically active forms into the effluent in sewage treatment plants. In addition, natural estrogens such as 17 β- estradiol (E2) and oestrone (E1), including their conjugates are excreted in the urine and faeces of humans and both are likely to contribute to the estrogenic properties of sewage effluent discharge (Larsson et al., 1999).

The zebrafish (*Danio rerio)* is a gonochoristic, undifferentiated species in which both sexes exist in an ovary- like stage. The males exhibit hermaphroditism initially before undergoing differentiation into the phenotypic sex (Takahashi, 1977; Chan and Yeung, 1983; Uchida et al., 2002; Maack and Segner, 2003). Consequently, the males could be sensitive to hormonal disturbances during this crucial period.
The sex organs of newly hatched zebrafish commence development as ovaries at 10-12 days post-hatch. In about 50 % of the juvenile fish, the ovaries continue to undergo development, while in the other 50 % of the population, the ovarian tissue regresses and the proliferation of the testicular tissue occurs (Hill and Janz, 2003).

The aim of the present study was to evaluate different sewage effluents obtained from an experimental sewage treatment plant situated at Hammarby Sjöstadsverket, Stockholm, Sweden. The efficiency of the various treatment processes conducted within the experimental pilot sewage treatment plant were assessed with the aid of a fish sexual development test (FSDT) and a reproduction test. Moreover, the potential toxicity of eight pharmaceuticals from different therapeutic classes were evaluated with the aid of a fish embryotoxicity test (FET) recommended by the Organisation of Economic Cooperation and Development (OECD).

# LITERATURE REVIEW

## Pharmaceuticals in the Environment

### *Occurrence*

The main sources of pharmaceuticals in the environment have been identified as human and veterinary applications (Heberer, 2002). Pharmaceuticals are usually excreted through faeces and urine in the form of a mixture of metabolites and original parent compounds (Sanderson et al., 2003). Subsequently, they enter municipal sewage treatment systems where they are either degraded, adsorbed to sewage sludge and eventually undergo dilution into surface water. Pharmaceutical compounds that adsorb to sludge usually gain access to the terrestrial environment when sludge is used as a fertilizer on farmlands (Fent et al., 2006). The hydrophobic and electrostatic interactions of a pharmaceutical with particulates and microorganisms determine its level of adsorption. Acetylsalicylic acid, ibuprofen, fenoprofen, ketoprofen, naproxen, diclofenac and indomethacin, a group of acidic non steroidal anti inflammatory drugs (NSAIDs), having pKa values ranging from 4.9 to 4.1, including clofibric acid, bezafibrate (pKa 3.6) and gemfibrozil exist as ion at neutral pH, and have little tendency of adsorption to sludge. Basic pharmaceuticals and zwitterions are capable of considerable adsorption to sludge and this has been observed in fluoroquinolone antibiotics (Golet et al., 2002).

Furthermore, drugs may occur in considerable concentrations in hospital waste water, waste water from manufacturers and landfill leachates (Holm et al.,1995; Kummerer, 2001; Richardson and Bowron, 1985). The direct application of pharmaceuticals in aquaculture (fish farming) (e.g. oxolinic acid), manure run-off , such as run-off following the use of sewage sludge and the application of manure on farmland as fertilizers are other sources of drugs in the environment (Halling-Sorensen et al.,1998). A number of non-prescription drugs from various therapeutic classes such as acetaminophen, caffeine, aspirin, ibuprofen, and controlled drugs such as carbamazepine, artorvastatin, gemfibrozil, fluoxetine, and 17 α-ethynylestradiol have been found at ng/L to µg/L levels in municipal wastewaters (Metcalfe et al., 2003;Boyd et al., 2003). The lipid lowering agents (bezafibrate, gemfibrozil, clofibric and fenofibric acids) have been detected in river waters at the nanogram per liter level (Stumpf et al., 1996; Ternes 1998). Diclofenac has been widely detected in sewage effluent in the µg/L range and in surface water at lower levels (e.g. Heberer and Stan, 1997; Ternes, 1998; Farre et al., 2001; Sedlak and Pinkston, 2001; Heberer, 2002).

Weigel et al., (2004) reported that ibuprofen and its metabolites occurred in samples of waste water and in seawater in Norway at concentrations of 0.1 – 20 µg/L (combination of ibuprofen and metabolites). Levels of ibuprofen up to 674ng/L and of naproxen up to 145ng/L were detected in two stormwater canals (Boyd et al.,2004).

In Canadian sewage treatment plants, naproxen was detected at a much higher level with median levels of 12.5 µg/L and maximal levels reaching 33.9 g/L (Metcalfe et al., 2003a).
Pharmaceuticals have been detected in drinking water on rare occasions (Heberer and Stan, 1996) and groundwater (Holm et al., 1995; Ternes et al., 2001). Concentrations of clofibric acid up to 165 ng/L (Stan et al., 1994) and 270 ng/L (Heberer et al., 1998) have been detected in tap water in Germany and the source has been attributed to the contamination of recharged groundwaters by sewage. The presence of various pharmaceuticals in German drinking water in the lower ng/L range, with a maximum of 70 ng/L for clofibric acid has been reported by several investigators (Stumpf et al., 1996; Ternes et al., 1999). The investigations performed indicated that the contamination of drinking water by pharmaceuticals does not constitute a widespread problem.

### *Metabolism*

It has been observed that pharmaceuticals that undergo renal excretion or are partially absorbed from the gut have the propensity of being eliminated from the body in their active forms (Ares, 1999). Consequently, a broad range of drugs, metabolites and their conjugates are disposed into the sewage system. The cytochrome P450 microsomal oxidase system has been identified as a principal route of formation of more polar and more readily excreted metabolites of various drugs (Daughton and Ternes,1999). Phase I and Phase II reactions are involved in the metabolism of pharmaceuticals. Phase I entails the use of monooxygenases, reductases and hydrolases for the addition of reactive functional groups to the molecules of drugs. On the other hand, Phase II involves the use of covalent conjugation in order to make the drug molecules hydrophilic and easily excretable (Daughton and Ternes, 1999).

The major forms in which pharmaceuticals are excreted are as unconjugated and conjugated polar metabolites (Fent, 2006). Studies have indicated that a high percentage of certain drugs can be conjugated (Forth et al., 1996; Ternes 1998) with conjugation being dependent on the chemical class of the drugs. The cleavage of conjugates of pharmaceuticals in sewage treatment plants (STP) has been observed to culminate in the release of the active parent compound in the case of estradiol and 17α-ethynylestradiol, the steroid hormone present in the contraceptive pill (Panter et al., 1999; Ternes et al., 1999; D'Ascenzo et al., 2003). Glucuronide conjugates (human metabolites) may undergo hydrolysis to yield the parent compound during waste water treatment or even in the environment (Roberts, 2005). The beta blocker, propranolol, undergoes extensive metabolism and less than 10% is excreted as the parent drug in faeces.

The persistence of carbamazepine in the aquatic environment could be attributed to the excretion of glucuronides which are capable of acting as a reservoir from which the parent drug could be released at a later time (Fent, 2006).

*Fate in the Environment*

The environmental fate and ecotoxicological impacts of numerous pharmaceuticals are not well comprehended and this is in contrast to other classes of substances (e.g. metals, PCBs, pesticides and nutrients) which have been studied extensively (Halling-Sorensen et al., 1998; Boxall et al., 2000; Jones et al., 2001). Detailed guidelines have been established for the procedure of assessment of pharmaceuticals in order to detect likely undesirable impacts on the environment (Fent, 2006). A draft guideline (Directive 2001/83/EC) was released by the European Commission specifying that an authorization for a pharmaceutical product intended for human use must be accompanied by an environmental risk assessment (EMEA, 2005).

The three major fates of pharmaceuticals in the environment include degradation to lower molecular weight compounds, physical sequestration by solids (culminating in removal as sludge), and hydrolysis of conjugates to produce the parent compound (e.g. clofibric and fenofibric acid conjugates) (Ternes, 1998). Adsorption to suspended solids (sewage sludge) and biodegradation are significant elimination processes in waste water treatment (Fent, 2006). The efficiency of elimination of pharmaceuticals from sewage treatment plants has been identified to be within the range of 0–99 % (Ternes, 1998; Stumpf et al., 1999; Carballa et al., 2004). The rate of elimination of a pharmaceutical may vary among different sewage treatment plants. Conditions such as floods, excessive water use and overflows as a result of sewage treatment plant failure could culminate in the direct release of untreated sewage into the environment (Velagaleti,1997). Ternes et al., (2002) remarked that sophisticated techniques such as ozonation, activated carbon or membrane filtration were efficient processes for the elimination of pharmaceuticals and other polar micro- pollutants from surface or municipal sewage effluents. Ozonation was identified as an efficient process of elimination of diclofenac from drinking water (Zwiener and Frimmel, 2000).

Biodegradation is considered to be the most crucial process of elimination of pharmaceuticals present to a large extent in the dissolved phase in waste water treatment plants. A large number of pharmaceuticals remain unaltered after being exposed to extensive degradation by microbes (e.g. mineralization) (Velagaleti, 1997). However, paracetamol (acetaminophen) yields products that are 58 and 25 times more toxic than itself following biodegradation (Bedner and MacCrehan, 2006). In activated sludge treatment, biodegradation can occur in aerobic (and anaerobic) zones, while the process is observed to occur anaerobically in sewage sludge digestion (Fent, 2006).

The biological decomposition of pharmaceuticals is enhanced with increase in hydraulic retention time and with age of the sludge in the activated sludge treatment. Diclofenac has been reported to be biodegraded to a considerable extent only when the sludge retention time was at least 8 days (Kreuzinger et al., 2004). Carbamazepine, a neutral drug which is not readily biodegraded, has been

observed to be poorly eliminated (normally less than 10 %), regardless of hydraulic retention times (Metcalfe et al., 2003a; Metcalfe et al., 2003b).

Buser et al., (1998b) identified photolysis as a significant process for the elimination of diclofenac in surface water. Studies have shown that direct and indirect forms of photolysis are crucial methods of elimination of propranolol (a β blocker), sulfamethoxazole (an anti bacterial), and ofloxacin (an antibiotic) (Andreozzi et al., 2003b). The anti epileptic drug, carbamazepine and the blood lipid regulator, clofibric acid are capable of undergoing slow photodegradation in salt and organic free water with half-lives of approximately 100days at latitudes of 50ºN in winter (Andreozzi et al., 2003b).
The intensity of the solar irradiation, characteristics of substances, presence of photosensitizers in water has been identified as factors that determine the efficiency of degradation of pharmaceutical substances by photolysis (Fent, 2006).

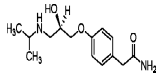
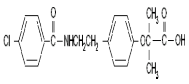
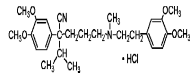
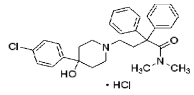
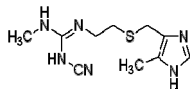
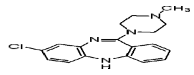
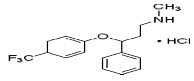
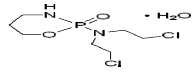
### *Effects on aquatic organisms*

Aquatic organisms have been identified as paramount targets of pharmaceutically active compounds (PhACs) since they might be continually exposed via wastewater residues throughout their entire life span (Fent et al., 2006).
Pharmaceuticals are evaluated for their acute toxicity with the aid of conventional standardized tests in conformity with well known and proven guidelines such as the Organization for Economic Cooperation and Development (OECD) and the International Organisation for Standardization (ISO). The approved laboratory organisms include algae, zooplankton, and other invertebrates and fish (Fent et al., 2006).

The non steroidal anti inflammatory drug (NSAID), acetaminophen, has been observed to inhibit the production of oestrogen-induced vitellogenin in isolated trout liver cells. The administration of 0.3mM acetaminophen produced an inhibition of the level of secreted vitellogenin to undetectable levels (Miller et al., 1999). Substantial changes in the plasma sex steroid levels were observed in the fish Japanese medaka, *Oryzias latipes* following exposure to propranolol for 14 days (Fent et al., 2006). Huggett et al., (2002) reported a reduction in the number of eggs released by the fish at 0.5µg/L after a 4- week exposure to propranolol at 0.5 and 1 µg/L. The decrease in the number of eggs released was attributed to a change in sex steroids and a subsequent reduction in oxytocin excretion.

A reduction in reproduction was induced in *Ceriodaphnia dubia* and *Hyalella azteca* following long term exposure to propranolol (Huggett et al. 2002). Reproduction was stimulated in *Daphnia magna* exposed to 36 µg/L fluoxetine for 30 days and in Ceriodaphnia dubia fertility was boosted at 56 µg/L (Flaherty et al., 2001 ), but was reduced in another study by Brooks et al., (2003).

Table 1. *Pharmaceuticals tested in the fish embryotoxicity test (FET). Data was obtained from Sigma Aldrich Website.*

| Drug | Molecular Formula | Molecular Weight | Chemical Structure |
|---|---|---|---|
| Atenolol | C14H22N2O3 | 266. 34 |  |
| Bezafibrate | C19H20CINO4 | 361. 82 |  |
| Verapamil hydrochloride | (CH3O)2C6H3 CH2CH2N(CH3)(CH2)3 [CH(CH3)2] CN.HCl | 491.06 |  |
| Loperamide hydrochloride | C29H33CIN2O2.HCl | 513.50 |  |
| Cimetidine | C10H16N6S | 252.34 |  |
| Clozapine | C18H19CIN4 | 326.82 |  |
| Fluoxetine Hydrochloride | C17H18F3 | 345.79 |  |
| Cyclophosphamide Monohydrate | C7H15Cl 2 N2O2 P.H20 | 279.10 |  |

*The modes of action of the various pharmaceuticals tested*

Fluoxetine (a prototype selective serotonin reuptake inhibitor) exerts its effects through the blockage of serotonin reuptake from the pre- synaptic nerve cleft (Ranganathan et al., 2001). Atenolol is a relatively specific beta 1 blocker that belongs to the same therapeutic category as propranolol, metoprolol and nadolol. However, bezafibrate, a blood lipid regulator acts mainly by decreasing serum triglycerides. The occurrence of bezafibrate in sewage effluent and surface water at 4.6 and 3.1 µg/L respectively has been reported (Stumpf et al., 1996; Ternes, 1998). Cyclophosphamide belongs to the group of antineoplastic drugs that act through interference with DNA replication, RNA transcription and replication and eventually the disruption of nucleic acid function. Verapamil is a slow- channel calcium blocking agent and a class IV antiarrhythmic drug. The antiarrhythmic features of verapamil have been attributed to its inhibitory effects on the cardiac conduction system. Clozapine is an atypical neuroleptic and a selective antagonist for D4 – dopamine receptor and it is increasingly used for the treatment of schizophrenia. Loperamide is a synthetic piperidine–derivative antidiarrhoel and it acts through the inhibition of gastrointestinal motility and excessive gastrointestinal propulsion. Cimetidine is an H2 receptor antagonist and anti- ulcer agent. It competitively inhibits histamine at the H2 receptors of the parietal cells.

# Endocrine disrupting chemicals (EDCs)

## Occurrence

Endocrine disrupting chemicals or endocrine disrupters are described as anthropogenic compounds that are capable of interfering with the endocrine system of vertebrates (Colborn et al., 1993). The main sources of EDCs in the aquatic environment are effluents from industries such as paper and pulp mills, agriculture, textile industries and sewage treatment plants. Persistent organochlorine pesticides (e.g., DDT, methoxychlor and dieldrin have been identified as endocrine-disrupting compounds. A variety of anthropogenic chemicals have been shown to act as EDCs, including high volume products such as phthalates, bisphenol A and alkylphenols (Sonnenschein and Soto 1998; Tyler et al., 1998; Vos et al., 2000).
EDCs exert their effects by mimicking or antagonizing endogenous hormones and by altering the synthesis and metabolism of endogenous hormones and hormone receptors (Sonnenschein and Soto, 1998).

Several studies have indicated the presence of natural (estradiol, oestrone) and synthetic (ethinyl estradiol) oestrogens, including xenoestrogens (e.g. nonylphenol, alkylphenols) in sewage effluents has been reported (Shore et al., 1993; Blackburn and Waldock, 1995; Stumpf et al., 1996). In Italian sewage treatment works, median effluent concentrations of 17β-estradiol (E2) at 1 ng/L and EE2 at 0.45ng/L have been detected (Baronti et al., 2000). Testosterone and androstenedione have

been detected in effluents from several domestic STW in USA (Kolodziej et al.,2003) and in pulp mill effluents (Durhan et al., 2002; Jenkins et al., 2003; Jenkins et al., 2001). The estimated concentrations of the androstenedione and the androstanedione in the effluent were 105 and 96 ng/L respectively (Thomas et al., 2002).

## Effects

Adverse health effects have been attributed to EDCs such as developmental, neurological, endocrine (Colborn et al., 1993) and reproductive alterations (Gray, 1998; Tyler et al., 1998; Janssen et al., 1998). Ethinyl estradiol has been identified to be the most potent oestrogenic substance while alkyl phenols are weak oestrogens (Purdom et al., 1994).

The exposure of zebrafish (*Danio rerio)* to 0.005 µg/L of ethinylestradiol has been associated with a delay in embryonic development (Kime and Nash, 1999).
The levels of the estrogenic biomarker protein vitellogenin (Vtg) were observed to be elevated in fish placed in cages downstream of STW (Purdom et al., 1994; Harries et al., 1996; Harries et al., 1997; Lye et al., 1997).

Studies have indicated widespread occurrences of intersex in wild roach (*Rutilus rutilus*) populations, which was more pronounced at the outlets of STW (Jobling et al.,1998).17α- ethinylestradiol (EE2), a synthetic steroid hormone present in contraceptive pills is persistent in the environment and elicits estrogenic activity in fish in the low ng/L range (Fent et al., 2006). Xenoestrogens have been reported to elicit morphological feminization of males and induction of the yolk protein precursor vitellogenin in fish (Smeets et al., 1999). Tyler et al., (1998) noted that EE2 is relatively persistent in the aquatic environment and it disrupts the endocrine system of fish. Gemfibrozil (a lipid lowering agent) has been identified as a potential endocrine disrupter in an in vivo study conducted in gold fish (*Carassius auratus*) (Mimeault et al., 2005).

Increased vitellogenin production has been reported in wild or caged fish following exposure to STW effluents (e.g. Folmar et al., 1996; Larsson et al., 1999; Svenson et al., 2002). Apart from domestic effluents, increased vitellogenin production has also been shown in connection with exposure to pulp mill (Mellanen et al., 1999; Tremblay and Van der Kraak 1999; Van den Heuvel and Ellis 2002) and refinery effluents (Knudsen et al., 1997). In Danish streams, sewage effluent load was found to positively correlate with roach intersex ratio (Bjerregaard et al., 2005). The phenomenon of intersex has been described in other wild fish species, such as barbel (*Barbus plebejus*), bream (*Abramis brama*), gudgeon (*Gobio gobio)* and carp (*Cyprinus carpio)* (e.g. Vigano et al., 2001; Vethaak et al., 2002; Allen et al., 1999). In roach exhibiting intersex, impaired reproductive capacity, reduced milt volume, decreased sperm density and reduced fertility have been observed (Jobling et al., 2002a ;Jobling et al., 2002b).

The androgen, 4- androstenedione and its metabolite 5α- androstanedione were identified as major contributors to the androgenic activity in a particular estuary receiving sewage effluent discharge (Thomas et al., 2002). In 1998, male- biased sex ratios were observed in the offspring of eelpout (*Zoarces viviparus*) that were sampled in the vicinity of a Swedish pulp mill (Larsson et al., 2000). In female guppies exposed to a 10% dilution of the same effluent under laboratory conditions, enhanced coloration was observed and this was thought to be an androgenic response (Larsson et al., 2002). In receptor -based in vitro assays, the androgenic effect of pulp and paper mill effluent discharges have been demonstrated (Svenson and Allard, 2004). In addition, effects such as a reduction in sex hormone levels and gonad size, elevated or decreased vitellogenin levels and delayed sexual maturation have been observed in fish exposed to effluent discharges from pulp and paper mills (Tremblay and Van Der Kraak 1999; Mellanen et al., 1999; Karels et al., 2001; van den Heuvel and Ellis 2002). The phenomenon of feminization in fish after exposure to high doses of androgens has been attributed to the aromatisation of the androgens into estrogens (Rinchard et al., 1999; Pifferer et al., 1993).

# Vitellogenin (Vtg)

The induction of vitellogenin (Vtg) is a commonly used endpoint for the detection of the effect of endocrine disrupters in fish (Tyler et al., 1996; Nichols et al., 2001; Tilton et al., 2001). Vitellogenin is a large serum phosphoglycolipoprotein that is synthesized by the liver in many oviparous organisms such as fish. The gene for Vtg exists in the liver of both females and males and it undergoes activation after exposure to oestrogen (Denslow et al., 1999). Vtg is transported via the blood to the gonads, where it is processed into lipovitellin and phosvitin, the nutrient sources of the developing embryo (Versonnen and Janssen, 2004). The synthesis of Vtg is regulated by the endocrine system under the control of feed back mechanisms through the hypothalamus-pituitary-gonadal-liver axis, 17β estradiol (E2) and testosterone. Levels of Vtg in the range of 10-20 mg/ml have been detected at peak activity in the plasma of females (e.g. Folmar et al., 1996; Parks et al., 1999).

17β -estradiol has been identified as the natural inducer of vitellogenin synthesis in the liver of female oviparous animals (Mommsen and Walsh, 1988). The maturational status of the female fish is usually indicated by plasma Vtg levels (Mommsen and Walsh, 1998; Arukwe and Goksøyr, 2003). Also, alkylphenolic compounds, phyto-oestrogens, synthetic oestrogens and some pesticides have been known to induce vitellogenin synthesis in males and females (Sumpter et al., 1996). In male or juvenile fish, the induction of vitellogenin has been acknowledged as an effective biomarker for the identification of estrogenic contamination in aquatic systems (Sumpter and Jobling, 1995). The production of Vtg in male fish can be induced by exposure to estrogenic substances (Sumpter and Jobling, 1995; Folmar et al., 1996; Tyler et al., 1996).

According to several studies conducted, an increase in vitellogenin production has been detected after exposure to different phytosterols e.g. b- sitosterol, genistein, biochanin A, equol and coumestrol (Latonnelle et al., 2002; Tremblay and Van der Kraak 1999; Mellanen et al., 1996 ; Pelissero et al., 1991). Different techniques have been developed to detect Vtg. These include radioimmunoassay, enzyme linked immunosorbent assay (ELISA), protein electrophoresis, western blot, northern blot and polymerase chain reaction (PCR) techniques (Heppell et al., 1995; Kramer et al., 1998; Nichols et al., 2001). The easiest assays are designed around ELISA techniques. The Western blot assay has been reputed to be very specific because the binding of the antibody to the appropriate protein after the separation of the protein by size can be observed easily (Denslow et al., 1999). However, it is difficult to carry out quantification for a large group of samples with the Western blot assay and hence, most laboratories have adopted the ELISA technique .

# Models for evaluating EDCs

The zebra fish *(Danio rerio)*, Japanese medaka (*Oryzias latipes*) and the fathead minnow (*Pimephales promelas*) have been proposed as model test species for the evaluation of EDCs (Ankley and Johnson 2004; OECD 2004). The endpoints approved for the evaluation of EDCs include vitellogenin, gonad differentiation, sex ratios and reproduction success.

The zebra fish *Danio rerio* (formerly *Brachydanio rerio*) is a tropical Cypriniform (family Cyprinidae) and its name is derived from its striped integument. It is a tropical freshwater fish found in the tributaries and branches of the Ganges River in South- East Asia (Eaton and Farley, 1974). Zebra fish is a small fish that adapts readily to different environments, has a short generation time and breeds throughout the year. This teleost cyprinid is characterized by high fecundity providing between 100 – 500 eggs at each spawning. The eggs are non-adherent, transparent and have a developmental period from fertilization to hatching of 96h at 26ºC (Laale, 1977). It is characterised by external development, transparency during organogenesis and tractable diploid genome which qualify it as a suitable model organism in research (Fishman et al., 1997). Complementary endpoints such as reproductive capacity, sex ratio and gonad morphology can be evaluated. This species of fish can be maintained easily and drugs can be administered to it with ease.

# The Fish Embryo Toxicity (FET) Test

This test was primarily developed for use with the zebra fish (*Danio rerio*) but it could also be adapted to other fish species recommended by the OECD such as fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) (Braunbeck et al ., 2005). The test entails the exposure of eggs from fertilisation until the completion of embryogenesis at 48 h post fertilisation (hpf). Exposure is usually conducted in well plates in small volumes of media. At constant intervals, individual monitoring of the embryos is carried out and the various observations are recorded (Schulte and Nagel, 1994).

Lethal and sub lethal endpoints have been identified (Nagel, 2002). The lethal endpoints include coagulation of egg, non detachment of the tail from the yolk, lack of somites and absence of heart beats. The completion of gastrulation, eye development, spontaneous movement, circulation, pigmentation, oedema and heart rate are regarded as sub-lethal endpoints that could be measured in order to determine the mode of action of the toxic response (Nagel, 2002).

The duration of the experiment could be prolonged in order to include measurements such as the presence of spinal deformations (Hollert et al., 2003), body or tail length (Nagel, 2002; Fraysse, Mons and Garric, 2006) and the pericardial area (Fraysse, Mons and Garric, 2006). The methodology has been employed in toxicity tests of environmentally relevant chemicals (Cook, Paradise and Lom, 2005; Fraysse, Mons and Garric, 2006; Kapp et al., 2006) and tests of sediment and sediment extracts (Hallare et al., 2005; Kammann, 2004).

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# Research Report

# The Toxicological Evaluation of Sewage Effluents and Pharmaceuticals with the use of Zebrafish as a Model Organism

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## Abstract

Aquatic organisms might be continually exposed to a complex mixture of substances in the form of sewage effluents throughout their lifespan. Several adverse effects such as endocrine disruption and a decrease in the reproductive potential of aquatic organisms have been attributed to the occurrence of pharmaceuticals and estrogenic substances in the aquatic milieu. Sewage treatment plants have been identified as the principal sources of environmental pollutants such as pharmaceuticals and endocrine disrupting chemicals in the aquatic environment, which highlights the importance of the development of efficient treatment processes. The present study was part of a large project that was conducted during a three week experimental period at a pilot sewage treatment plant (STP) situated in Stockholm, Sweden. The main focus of the project was to evaluate different treatment processes for the reduction of selected pharmaceuticals and estrogenic chemicals.
In the present study, the sewage effluents were evaluated using a fish sexual development test and a reproduction test with zebrafish (*Danio rerio*) as a model test species. Several of the effluents caused an increase in vitellogenin production, as well as effects on gonad maturation, spawning ability and egg laying. Both the analytical chemistry and the tests with zebrafish indicate that traditional treatment techniques are not capable of sufficiently reducing the levels of pharmaceuticals and estrogens, while ozonation was the most efficient treatment used at the pilot STP. Furthermore, eight different pharmaceuticals viz Clozapine (anti psychotic), Atenolol (β-blocker), Cimetidine (anti histamine), Fluoxetine (anti depressant), Loperamide (anti diarrhoeal), Verapamil (calcium channel blocker), Bezafibrate (lipid lowering agent) and Cyclophosphamide (anti neoplastic) were evaluated for toxicity in zebrafish embryos with the use of an OECD recommended fish embryotoxicity test. The endpoints monitored included hatching time, heart rate, tail extension, circulation, coagulation, spinal deformation, death and oedema. All the pharmaceuticals were tested at 5 different concentrations ranging from 1µg/L to 10mg/L. The only significant effects observed were a decrease in the heart rate in zebrafish embryos exposed to the highest concentration of Clozapine and abnormalities in the embryos exposed to all the concentrations of Clozapine. For the pharmaceuticals, the fish embryotoxicity test appears to be less sensitive than for other types of chemicals that have been tested in previous studies.

Keywords: Zebra fish, *Danio rerio*, pharmaceuticals, reproduction test, fish sexual development test, vitellogenin

# Introduction

The emergence of environmental pollutants has been an issue of ongoing concern. Several adverse effects have been observed in terrestrial and aquatic organisms exposed to various contaminants in the environment. Furthermore, the reproductive potential of aquatic organisms has been threatened by the presence of substances capable of influencing the hormonal system. It is apparent that aquatic organisms are continually exposed to a diverse mixture of chemicals originating from industrial, agricultural and domestic sources throughout their lifespan.

Effluents from municipal sewage treatment plants have been demonstrated to contain a complex mixture of substances including endocrine disrupting chemicals (EDCs) and pharmaceuticals. The natural estrogens, 17 β- estradiol (E2) and estrone (E1), including the synthetic contraceptive pill hormone, 17α- ethinyl estradiol (EE2) have been identified as the main estrogenic substances that occur in sewage treatment plant effluents (Desbrow et al., 1998). In addition, estrogens have been detected in the environment in the lower ng/L range. In Sweden, levels of E1, E2 and EE2 ranging from 1- 6 ng/L were detected in waste water from a sewage treatment plant (Larsson et al., 1999).

Sewage effluents have been observed to be estrogenic to fish according to several reports (Purdom et al., 1994; Harries et al., 1997; Larsson et al., 1999; Svenson et al., 2002). Moreover, several investigations conducted have indicated various effects in fish exposed to pulp and paper mill effluents such as a reduction in the levels of sex hormones and gonad size, increased or decreased vitellogenin levels and delayed sexual maturation (e.g. Tremblay and Van der Kraak 1999; Mellanen et al., 1999; Denslow et al., 2004). The exposure of fish to EE2 has been associated with the induction of vitellogenin, alterations in sex ratios, the suppression of the development of the sexual organs and intersexuality (Blazquez et al., 1998; Papoulias et al., 1999; Scholz and Gutzeit, 2000; Metcalfe et al., 2001 ; Van Den Belt et al., 2002). Intersexuality has been documented in Japanese medaka (*Oryzias latipes*) exposed to nonylphenol (NP), octylphenol and o,p´ - DDT (Gray and Metcalfe, 1997; Gray et al., 1999b; Metcalfe et al., 2000). In addition, female mosquito fish (*Gambusia affinis*) in the vicinity of a paper mill in Florida, USA were observed to portray male sexual characteristics ( e.g. Howell et al., 1980; Jenkins et al., 2001). Male- biased sex ratios were observed in eelpout (*Zoarces viviparus*) sampled in the vicinity of a Swedish pulp and paper mill (Larsson and Forlin, 2002).

Pharmaceuticals have been mainly detected in the environment in proximity to sewage treatment plants, in waste water and in surface water in most countries (Halling-Sorensen et al ., 1998; Kolpin et al., 2002; Gross et al., 2004).
Furthermore, most pharmaceuticals have been reported to occur in waste water effluents in the ng/L to µg/L range (Fent et al., 2006). Pharmaceuticals are

biologically active compounds that have been identified as potent water pollutants (Jorgensen and Halling- Sorensen, 2000; Daughton and Ternes, 1999). The concentrations of pharmaceuticals detected in the environment are usually in the ng/L - µg/L range (Daughton and Ternes, 1999). Studies conducted indicated that pharmaceuticals occur in waterbodies such as rivers, lakes and seawaters in the ng/L range (Kolpin et al., 2002; Ashton et al., 2004; Thomas and Hilton, 2004).
The main sources of pharmaceuticals in the environment have been identified as human and veterinary applications (Heberer, 2002). These group of compounds are usually excreted through faeces and urine in the form of a mixture of metabolites and original parent compounds (Sanderson et al., 2003). Subsequently, they enter municipal sewage treatment systems where they are either degraded, adsorbed to sewage sludge or eventually undergo dilution into surface water. Pharmaceuticals such as the beta blockers propranolol, bisoprolol and metoprolol have been detected at peak levels of 0.59, 2.9 and 2.2 µg/L respectively in surface water (Ternes, 1998). Clofibric acid has been detected in ground water at 4µg/L ( Heberer and Stan, 1997) and in drinking water at 0.07 - 0.27µg/L (Stumpf et al., 1996 and Heberer and Stan, 1997). The anti neoplastic drugs, ifosfamide and cyclophosphamide have been found at concentrations up to 4.5µg/L in hospital effluents (Steger – Hartmann et al., 1997), and at ng/L in municipal waste water ( Kümmerer et al ., 1997 and Steger – Hartmann et al., 1997).

Pharmaceuticals have been reported to exert various effects on organisms. In the Japanese rice fish (*Oryzias latipes*), a reduction in the numbers of eggs produced and hatched was detected following exposure to propranolol (a β blocker) at 5µg/L for 4 weeks (Carlsson et al., 2006). An elevation of plasma oestradiol levels in female fish and developmental abnormalities of fish embryos were observed following exposure to 0.1 and 0.5 µg/L of fluoxetine respectively (Brooks et al., 2003a). In another investigation, abnormalities such as oedema, curved spine, incomplete development (absence of pectoral fins, microophthalmia and nonresponsiveness) were observed in the developing embryos of the Japanese medaka (*Oryzias latipes*) exposed to fluoxetine at 0.1, 0.5, 1.0 and 5.0 µg/L for a duration of 4 weeks (Brooks et al., 2003).

It is apparent from several studies that have been conducted that environmental pollutants such as sewage effluents and pharmaceuticals are capable of producing effects in the aquatic environment and therefore they should be closely monitored in the environment.
The aim of the present study was to evaluate the different sewage effluents obtained from an experimental sewage treatment plant situated at Hammarby Sjöstadsverket, Stockholm, Sweden. The efficiency of the various treatment processes conducted within the experimental pilot sewage treatment plant were assessed with the aid of a fish sexual development test (FSDT) and a reproduction test. Moreover, the potential toxicity of eight pharmaceuticals from different therapeutic classes were evaluated with the aid of a fish embryotoxicity test (FET) recommended by the Organisation of Economic Cooperation and Development (OECD).

# Materials and Methods

## Sewage Treatment Plant (STP) effluents studied

The sewage effluents studied were obtained from the pilot sewage treatment plant (STP) situated at the Hammarby Sjöstadsverket, Stockholm, Sweden. The present study was part of a large project involving several studies that was conducted during a three week experimental period at the STP. The pilot STP is composed of four different experimental process lines in which the first and second lines are based on aerobic processes as the main biological treatments, with activated sludge and a membrane reactor, respectively. Line 1 has the same process layout as the main existing central STP in Stockholm (Henriksdals STP). Lines 3 and 4 have anaerobic processes as principal biological treatment techniques. Incoming untreated sewage water is the same as for Henriksdals STP. The sewage effluents evaluated in the present study were obtained from Line 1 and Line 2. Six different treatment processes were evaluated, viz A2- A7 (Figure 1). The sewage effluents from the different treatment steps from Line 1 were denoted A2- A6, while Line 2 was denoted A7. A1 was used as the clean reference water and it was composed of charcoaled tap water with the addition of 2.5% sewage from A3. A2 was subjected to 'After sedimentation' treatment. A3 is the outgoing water from Line 1 (corresponding to Henriksdals STP) and it has a sandfilter coupled after the 'After sedimentation' treatment. A4 has a sandfilter and a biofilter in combination with the 'After sedimentation' treatment. A5 has a sandfilter and an ozonation step in addition to the 'After sedimentation' treatment. A6 has a sand filter, ozone and a biofilter in addition to the 'After sedimentation' treatment. A7 is the outgoing water from Line 2, which consists of a filtering drum and a membrane bioreactor.

The sewage effluents were obtained from Hammarby Sjostad's STP in April 2007 and transported to the Department of Biomedical Sciences and Veterinary Public Health, SLU. Grab samples were taken in 25 litres plastic containers every third day during the three weeks experimental period. Subsequently, they were frozen and stored at -20ºC. The effluent samples were thawed overnight in room temperature prior to use.

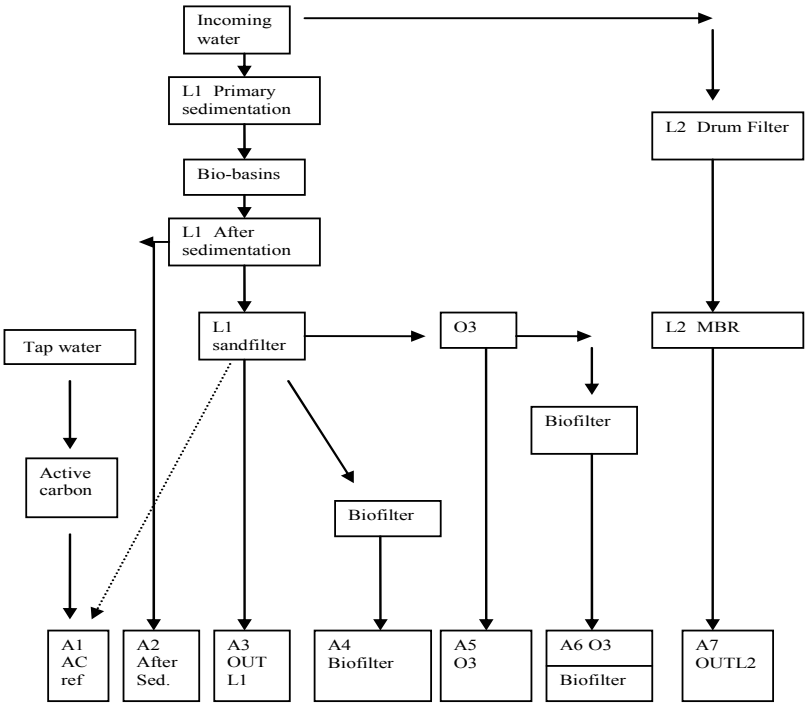



**Figure 1**. *Outline of the treatment processes at Hammarby Sjostad's Sewage treatment Plant. L1 denotes the first line, L2 denotes the second line, AC ref denotes active carbon reference water, OUTL1 denote line one outgoing water, O3 denotes ozone treatment while MBR and OUTL2 denote Membrane Bioreactor and the second line outgoing water respectively. The A2 - A7 connote the different sewage effluents tested. (Chart by Bjorlenius, 2007).*

## Chemical analyses

Incoming sewage water and the different treatment steps were analyzed for contents of selected pharmaceuticals (Laven et al., manuscript in preparation) and selected estrogenic substances (Adolfsson-Erici et al., manuscript in preparation).

## Experimental animals

Adult zebra fish (*Danio rerio*) were procured from a local supplier in Uppsala, Sweden and were allowed to adapt to laboratory conditions for 4 weeks before the commencement of the project. The study was conducted at the Department of Biomedical Sciences and Veterinary Public Health, SLU.
The animals were kept in aquaria within a temperated laboratory at 26 ± 1ºC and a 12- h light / dark cycle was maintained. Standardised water (ISO 7346- 1, 1996) was used for the maintenance of the adult fish. It was prepared from deionised water and the following salts were added : $CaCl_2$ x $2H_20$ (117.6 mg/l), $MgS0_4$ x 7 $H_20$ (49.3 mg/l ), $NaHC0_3$ (25.9 mg/l ) and KCl (2.3 mg /l) (Sigma Aldrich Sweden AB). The adult fish were fed commercial flake food (Sera®) and freeze dried chironomids (Nutrafin®) 2-3 times daily.

## Fish sexual development test (FSDT)

Adult males and females were transferred to cone shaped breeding funnels for spawning. The funnels were filled with 20-25 litres of standardised water. Adult male and female fish were placed in a ratio of 2:1. Spawning substrates (glass marbles) were placed in the reproduction funnels in order to facilitate breeding. The eggs in the breeding funnels were collected the following day between 30 and 60 minutes after light was turned on in the laboratory. The eggs were subsequently transferred into a 10 liter aquarium containing standardized water. The embryos were raised until 20 days post hatch after which they were transferred to 8-litre aquaria containing the different sewage effluents.
The zebrafish juveniles were exposed to the sewage effluents (A1- A7) as well as standardized water (A8) from 20-60 days post hatch (dph). Each group (A1- A8) consisted of two replicates (A and B) with 40 juveniles each. The fish were fed commercial flake food (Sera ®, Tetra ®) and freeze-dried red grubs 2-3 times daily. Exposure was conducted through a flow through system with a 25% daily renewal of the exposure media, which was maintained with the aid of a multichannel peristaltic pump (Ismatec®, Zurich Switzerland). The effluents were pumped from the 25-L plastic containers via glass capillaries connected with silicon tubings to the aquaria. Standardised water was pumped from a stainless steel tank. The experiment was conducted at 26ºC and in a 12-h light /dark cycle. At 60 days post hatch (dph), eight males were sampled from each replicate for vitellogenin analysis (description below). The remaining fish were euthanized in MS 222 (1g/L) and then fixed and processed for histological evaluation of gonad morphology and sex determination (description below).

## Reproduction test

Groups of adult zebrafish were placed in 30-litre aquaria containing the different sewage effluents (A1- A7) and the standardized fish water (A8). For each effluent tested, four replicates where used. The fish were placed in stainless steel net spawning cages within each aquarium and each replicate consisted of five fish (three males and two females). Effluent water was pumped from the 25-L plastic containers to each aquarium with the use of a multichannel peristaltic pump (Ismatec®, Zurich Switzerland) through glass capillaries connected with silicon tubings. Standardised water was pumped from a stainless steel tank. The flow rate in the aquaria corresponded to a 20% daily renewal of the exposure media. The experiment was conducted at 26ºC and in a 12-h light /dark cycle. Each morning the eggs from each replicate were collected by siphoning with silicon tubing from collecting containers that were placed under each spawning cage. The duration of the exposure of the fish to the effluents was 21 days. The endpoints monitored on a daily basis were spawning (yes/no), number of eggs (fecundity) and fertilization of the eggs. The eggs were examined with the aid of a stereo microscope in order to determine whether they were fertilized or not. For groups with a large number of eggs, 40 eggs were selected for the evaluation of the number of fertilized eggs. At the end of each exposure week, i.e. after 7, 14 and 21 days of exposure, fish embryotoxicity tests were conducted for the exposed groups in accordance with the fish embryotoxicity test (FET) described separately. At the end of the exposure, the male zebra fish from each replicate were anaesthetized in tricaine methane sulfonate (MS 222 (1g/L) and then sampled for vitellogenin analysis. Subsequently, the female fish were euthanized and then fixed and processed for histological evaluation of gonad morphology.

## The Fish Embryotoxicity Test (FET)

The assay is based on a method described by Schulte and Nagel (1994) and with contributions from Carlsson and Norrgren (2004). The eggs were obtained as previously described under the fish sexual development test (FSDT). The pharmaceuticals studied were clozapine (an anti psychotic), fluoxetine (an anti depressant),bezafibrate (a lipid lowering drug), cyclophosphamide (an antineoplastic), cimetidine (an anti histamine), loperamide (an antidiarrhoel), verapamil (a calcium channel blocker) and atenolol (a β blocker). The pharmaceuticals were selected based on their occurrence and potential toxicity to the aquatic environment (Sanderson et al. 2003).

Stock solutions of the pharmaceuticals were prepared in dimethylsulphoxide (> 99.5% DMSO; Merck,) and then diluted 1:1000 in standardised water to exposure concentrations of 1µg/L, 10 µg/L, 100 µg/L, 1 mg/L and 10 mg/L. Standardised water and DMSO were used as negative controls. The concentration of the carrier DMSO was 0.1% in the final test and negative control solutions. A mixture of

musk ketone 50µg/L (Sigma Aldrich Sweden) and 7.6mg/L phenylthiourea (Sigma Aldrich Sweden AB) was used as positive control for decrease in heart rate and inhibition of pigmentation respectively. Newly laid eggs were placed in Petri dishes containing the test solutions.
The eggs were examined in a stereo microscope for the selection of eggs that had reached the four-cell stage (OECD 2006). For each pharmaceutical and exposure concentration 12 eggs were selected and transferred individually to 96 micro-well plates (Costar, Corning Incorporated, USA) with 250 µL of the test solutions. The plates were covered with Para film and then kept at 26ºC and in a 12-h light /dark cycle. The examination of the eggs was carried out with the aid of a stereo microscope at 24, 48 and 144 hours post fertilization (hpf). In order to prevent thermal stress to the embryos, microscopic examination was performed with a light source equipped with a fiber optic cable.
Photos of the micro-well plates were taken with a mounted digital interval timer camera (Canon Power Shot Pro 1) once every hour from 48 to 144 hours.

The various endpoints monitored include tail extension (24h), coagulation (24 and 48h), spontaneous movement (24h), heart rate (48h), circulation (48h), spinal deformation (144h), pigmentation (24h), oedema (48h), eye development (24 and 48h) and hatching time (144h). The tail extension of the zebra fish embryos was measured on a scale of 1- 3, where one denoted the complete detachment of the tail from the yolk sac, two denoted partial extension while three denoted lack of detachment of the tail from the yolk sac. The heart rate was determined by counting the number of heart beats during 30 seconds. The circulation was observed as the flow of blood in the caudal artery. The spinal deformation of the zebra fish embryos was measured on a scale of 1-4, where one denoted the absence of deformation, two and three denoted curvature at less than 45º and greater than 45º respectively while four denoted curvature at several locations. The pigmentation of the embryos was measured on a scale of 1-4, where one denoted full pigmentation, two and three denoted a reduction in the intensity of pigmentation while four denoted the absence of pigmentation. Coagulation, circulation, eye development and oedema were measured as categorical data (yes or no basis).

## Vitellogenin analysis

The heads and tails of the sampled fish were cut, weighed and placed in 1.5 ml eppendorf tubes. The heads and tails were removed for vitellogenin analysis while the bodies were used for the confirmation of the sex of each fish. Subsequently, the head and tail samples were cut, weighed, placed individually in 1.5 ml eppendorf tubes, and then immediately frozen in liquid nitrogen and stored at -80ºC. The sampled fish were homogenized individually in buffer (Tris-HCl (Tris-Ultra Pure ® (ICN, Denmark) pH 7.4 + 1% Protease inhibitor cocktail, Sigma®) with the aid of a manual homogenizer.
The homogenate was centrifuged at 13000xg at 4º C for 30 minutes and the supernatant below the fat layer was collected to determine the vitellogenin concentration of each fish. The supernatants were aliquoted and frozen at - 80ºC. The measurement of Vtg was conducted by using a commercially available pre-coated vitellogenin ELISA kit (Biosense laboratories ®, Norway). Purified vitellogenin from zebrafish was used as a standard. The procedure was conducted according to the manufacturer's instructions. The absorbance was measured using a microtiter plate reader (Lab systems Multiskan MS, Finland) and the concentration of VTG in each fish was calculated.

## Histological preparation and evaluation

The specimens (the bodies of the fish) intended for histological evaluation were placed in individually labelled plastic cassettes. After dehydration in 70% absolute ethanol, the specimens were treated with xylene and finally embedded in paraffin. Each paraffin block contained 6- 10 individuals. The paraffin blocks were sectioned longitudinally in a dorsal-ventral position. The paraffin blocks containing the tissues were sectioned on a microtome at about 3 - 5 microns thin sections. The sections were transferred to glass slides and placed on a heating plate for one hour in order to allow them to settle by drying. The sections were deparaffinised with xylene and rehydrated using a graded series of ethanol and finally tap water was added in order to prepare the sections for staining with hematoxylin and eosin. Following staining, the sections were dehydrated again in ethanol and xylene and then mounted with cover slips. The gonads of the females were classified depending on the level of maturity as stages 1 and 2 ovaries. The ovaries that contained oocytes at the primary growth stage and oocytes that contained cortical alveoli were classified as Stage I ovaries. The vitellogenic stage (Stage II ovaries) were classified based on the presence of oocytes that contained yolk vesicles. In the males, the gonads were classified as stages 1 and 2 , depending on the amount of spermatozoa in the lumen. The lumen that contained little or approximately 10% spermatozoa were classified as Stage 1, while those that contained more than 10% spermatozoa were designated as Stage 2 gonads.

# Statistics

The sex ratios and gonad maturations (FSDT test), as well as successful spawning occasions (Reproduction test) were tested for differences between exposed groups (A2-A7) and controls (A1) using the Chi square test, with subsequent Bonferroni - Holm adjustment of p-values. The data on number of eggs laid and fertilization were analyzed with one–way analysis of variance (ANOVA) followed by Dunnett's post-hoc test for comparing exposed groups with controls. Differences in vitellogenin concentrations between exposed groups and controls were tested using the non-parametric Mann-Whitney U test. The data of the group replicates were combined if no differences were detected between the replicates. In the FET tests, data were analyzed by one-way ANOVA followed by Dunnett's post-hoc test for comparison of exposed groups with controls (DMSO carrier). The transformation of the data was performed whenever it was necessary in order to fulfil the requirements of ANOVA. Categorical data such as coagulation, circulation and eye formation were analysed with the Chi square test. The software used for analyzing the data were Statview 5.0.1 (SAS Institute Inc.) and MINITAB release 14 (Minitab Inc.). The level of significance was set at 0.95 ($p<0.05$). Data is presented as mean ±standard deviation (SD) unless otherwise stated.

# Results

## Fish Sexual Development Test (FSDT)

Increased vitellogenin concentrations were measured in fish exposed to the A2, A3 and A4 effluents (Fig. 2). No statistical differences in sex ratios were observed between exposed groups and the control group (Fig.3). Generally, although, there were more females present in exposed groups, in particular A2, A3 and A4 (45-47%), as well as in the internal control A8 (46%) compared with A1 controls (35%). No intersex fish were observed in all of the groups. In all exposed groups the gonad maturation of males was different from the control group (Fig.4). A large proportion of the males in the control group were classified as maturing (Stage 1), while in the exposed groups, as well as the A8 group, the males were predominantly mature (Stage 2). More females in the A7 group were classified as immature compared with controls (Fig. 5).

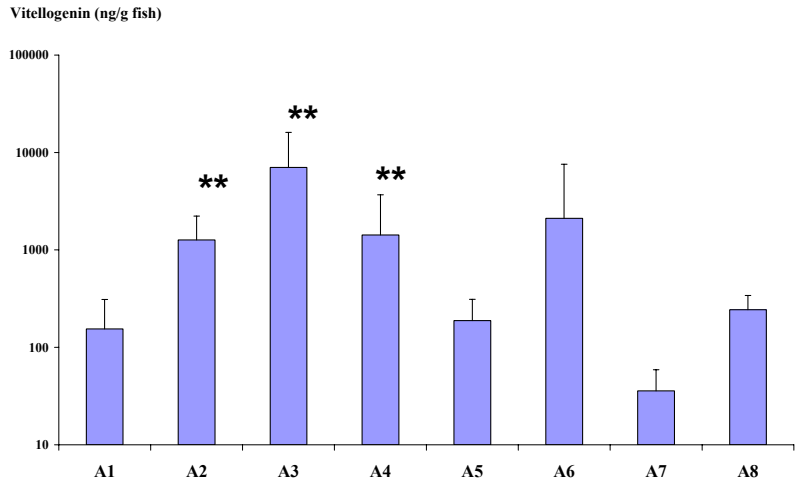



**Figure 2**. *Mean (± S.D.) vitellogenin concentrations at 60 days post hatch (dph) in zebrafish exposed to sewage effluents (A1- A7). A8 was used as an internal control. ** indicate significant differences at p < 0.01 level.*

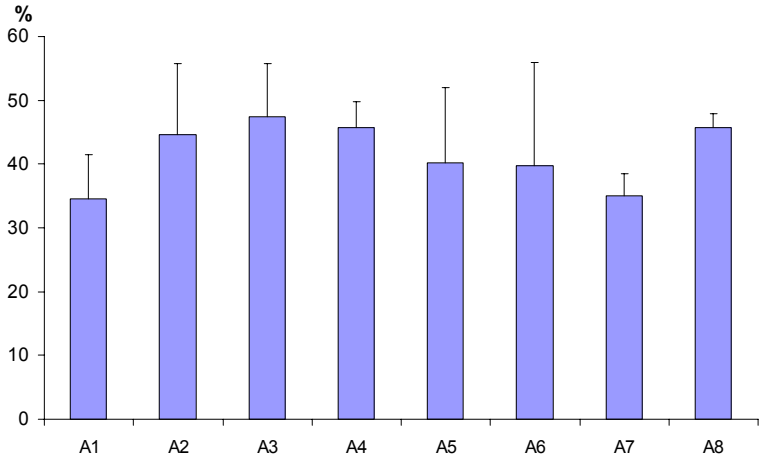



**Figure 3**. *Mean percentages of females in groups of zebrafish exposed from 20 to 60 days post-hatch to different sewage effluents (A1 - A7). A8 was used as an internal control.*

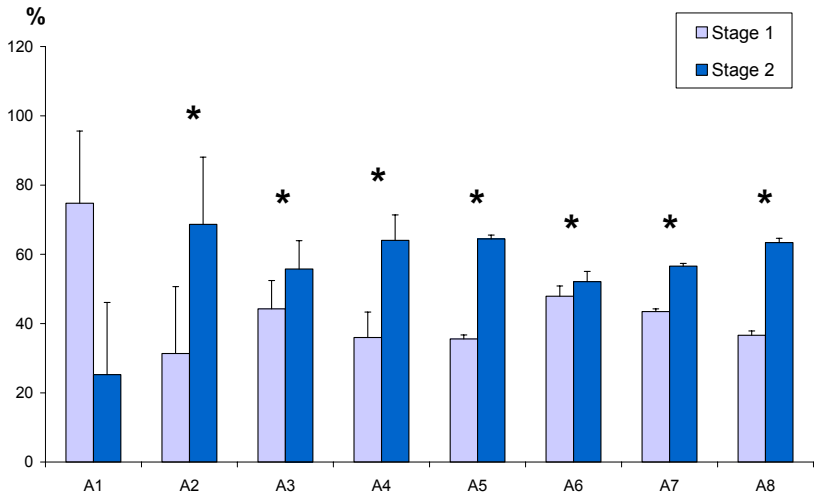



**Figure 4**. *Gonadal maturation in male zebrafish exposed to sewage effluents (A1-A7). A8 was used as an internal control. The number of males was depicted in the form of percentages as maturing (Stage 1) and mature males (Stage 2) at 60 days post hatch (dph). * indicates significant differences at $p < 0.05$ level.*

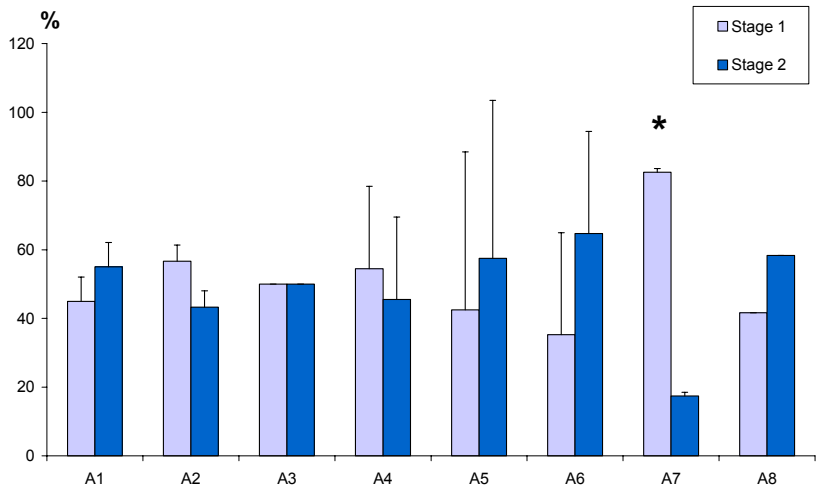



**Figure 5**. *Gonadal maturation in female zebrafish exposed to sewage effluents (A1- A7). A8 was used as an internal control. The number of females were depicted in the form of percentages as immature (Stage 1) and maturing (Stage 2) at 60 days post hatch (dph). * indicate significant differences at $p < 0.05$ level.*

## Reproduction test

Significant differences were observed in the spawning success of zebrafish exposed to sewage effluents. Fish exposed to effluent A2 had a higher number of successful spawnings than controls, while fish in groups A3, A4 and A5 exhibited a decrease in spawning ability (Fig. 6). Fish exposed to effluent A4 produced a fewer number of eggs per spawning compared with controls (Fig. 7). There were no differences in the number of fertilized eggs between the exposed groups and controls. There were no significant differences in vitellogenin concentrations in the male fish exposed to sewage effluents compared with controls. The gonads of the male and female adult zebrafish exposed to the sewage effluents were fully developed, with no differences observed between exposed groups and controls.

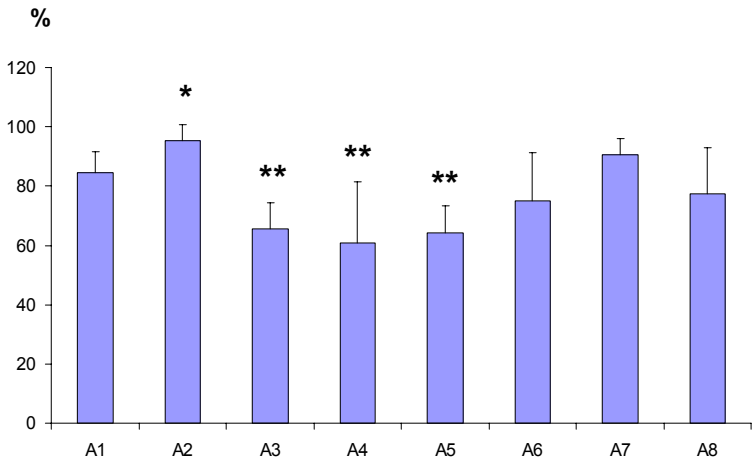



**Figure 6**. *Percentages of successful spawning occasions in zebrafish exposed to different sewage effluents for 21 days. ** indicate significant differences at p< 0.01 level.*

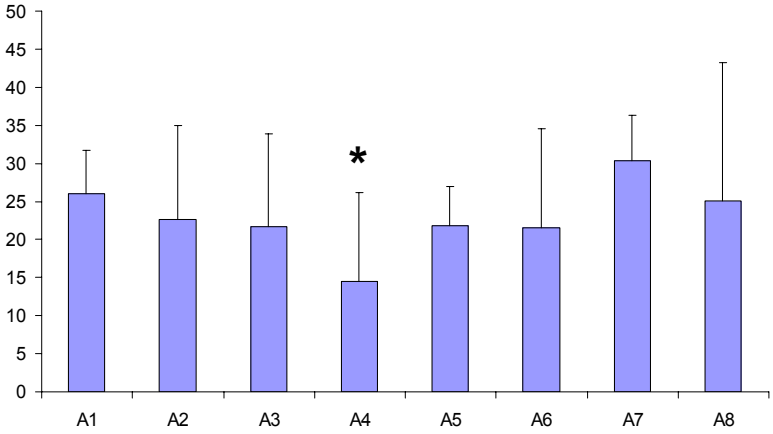



**Figure 7**. *The mean number of eggs/spawning in zebrafish exposed to different sewage effluents for 21 days.*

## The Fish Embryotoxicity Test (FET)

Of all the pharmaceuticals studied, only clozapine elicited significant effects on the zebrafish embryos. Several abnormalities (such as malformations, eye abnormalities and scoliosis) were observed in embryos exposed to all the concentrations of clozapine. A decrease in the heart rate was elicited in the embryos exposed to the highest concentration of clozapine (Fig. 8). In addition, a reduction in viability was observed in the zebrafish embryos exposed to the highest concentration of clozapine. No significant effects were observed in the FET conducted on the sewage effluents (A1- A7).

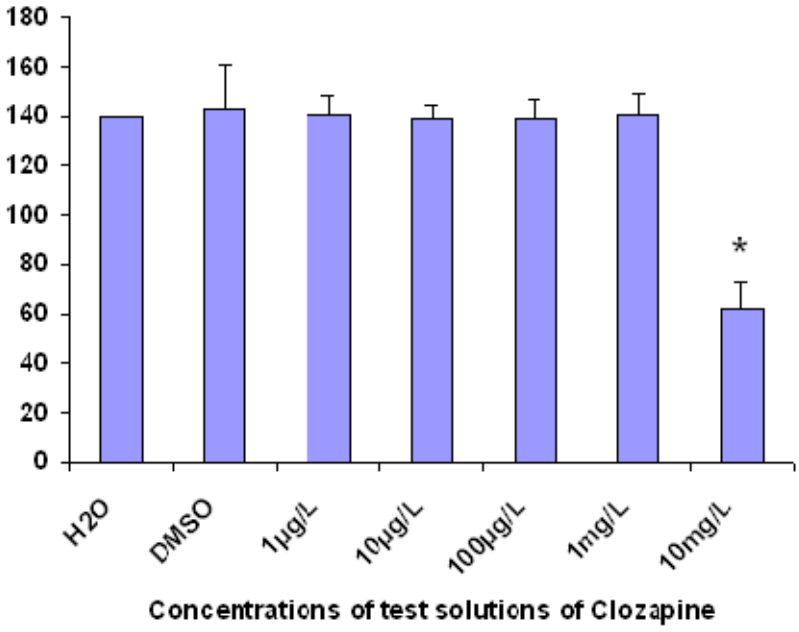
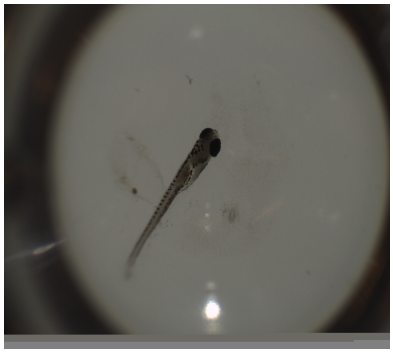
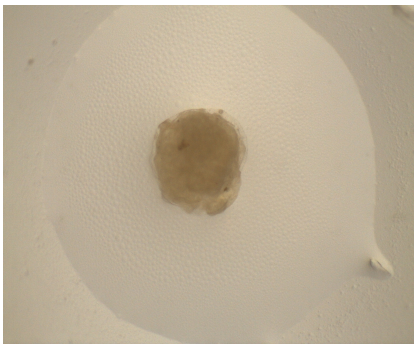



**Figure 8**. *Heart rate (beats per minute) in zebrafish embryos exposed to clozapine with 12 embryos per concentration. The heart rate was monitored at 48 hours post fertilization (hpf).*





A. B.

**Figure** *(A). Normal embryo at 144 hpf (B). Coagulated embryo at 24 hpf. Both were observed following exposure to Clozapine at 10mg/L.*

## Chemical analyses

The concentrations of selected pharmaceuticals and estrogenic substances analysed in the incoming water and the treatment steps are presented in Table 2.

**Table 2**: The sewage effluents in the Stockholm water pharmaceutical project were analysed in order to determine their composition. The concentrations are expressed in µg/l for the pharmaceuticals and in ng/L for the estrogenic chemicals (Laven et al., Adolfsson- Erici et al., manuscripts in preparation). A1 denotes the reference water (charcoaled tap water + 2.5% sewage), A2 (after sedimentation treatment), A3(Outlet L1), A4 (Biofilter), A5 (Ozone), A6 (Biofilter + Ozone) and A7 (Outlet 2).

| **Pharmaceuticals (µg/L)** | **Incoming water** | **A1** | **A2** | **A3** | **A4** | **A5** | **A6** | **A7** |
|---|---|---|---|---|---|---|---|---|
| Atenolol | 1.4 | 0.045 | 0.80 | 1.1 | 0.70 | <0.05 | 0.052 | 0.22 |
| Carbamazepine | 0.28 | 0.013 | 0.36 | 0.41 | 0.31 | <0.01 | <0.01 | 0.36 |
| Cyclophosphamide | <0.015 | <0.01 | <0.010 | <0.01 | <0.010 | <0.01 | <0.01 | <0.01 |
| Diclofenac | 0.17 | <0.01 | 0.52 | 0.11 | 0.44 | <0.01 | 0.020 | 0.23 |
| Enalapril | 0.23 | <0.01 | <0.01 | <0.01 | <0.010 | <0.01 | <0.01 | <0.01 |
| Gemfibrozil | 0.29 | <0.02 | 0.37 | 0.18 | 0.32 | <0.01 | <0.01 | <0.01 |
| Hydrochlorothiazide | 1.1 | <0.02 | 1.5 | 1.2 | 1.7 | <0.02 | <0.02 | 1.2 |
| Ibuprofen | 7.7 | <0.03 | 0.34 | 0.38 | 0.067 | 0.038 | <0.03 | <0.03 |
| Ketoprofen | 1.2 | <0.01 | 0.37 | 0.66 | 0.27 | 0.042 | 0.020 | 0.042 |
| Metoprolol | 0.77 | <0.02 | 0.66 | 0.80 | 0.64 | <0.03 | <0.03 | 0.34 |
| Naproxen | 3.2 | <0.03 | 0.35 | 0.35 | 0.11 | <0.02 | <0.02 | 0.075 |
| Oxazepam | 0.40 | <0.01 | 0.45 | 0.54 | 0.45 | 0.051 | 0.047 | 0.45 |
| Paracetamol | 84 | <0.03 | <0.02 | <0.02 | <0.020 | <0.02 | <0.02 | <0.02 |
| Propanolol | 0.087 | <0.00 | 0.080 | 0.090 | 0.084 | <0.00 | <0.005 | 0.094 |
| Ranitidine | 0.53 | <0.02 | 0.13 | 0.34 | 0.17 | <0.02 | <0.02 | 0.14 |
| Terbutaline | <0.020 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| **Estrogenic Chemicals (ng/L)** | **Incoming water** | **A1** | **A2** | **A3** | **A4** | **A5** | **A6** | **A7** |
| Ethinylestradiol | 0.90 | 0.060 | 0.200 | 0.200 | 0.070 | 0.040 | 0.030 | 0.100 |
| Estrone | >10 | 0.055 | 8 | 0.700 | 0.080 | 0.150 | 0.060 | 0.700 |
| Estradiol | 9 | 0.040 | 0.300 | 0.200 | 0.150 | 0.040 | 0.030 | 0.040 |
| Bisphenol A | 1600 | 0 | 700 | 400 | 100 | 0 | 0 | 0 |
| Triclosan | >100 | <10 | 100 | 75 | 30 | 0 | 0 | 12.5 |

# Discussion

Waste water treatment plants have been identified as a major source of anthropogenic substances in the environment (Hill and Janz, 2003). Consequently, effluent discharges consisting of complex mixtures of natural and synthetic hormones, alkylphenols, phthalates, pharmaceuticals and certain pesticides are released into the environment from the sewage treatment plants (Giger et al., 1984; Ternes et al., 1999; Kolpin et al., 2002). The transformation processes for pharmaceuticals in a sewage treatment plant and the influencing factors include the composition of the sewage, weather conditions, the design and operation of the treatment process (Ternes, 1998; Johnson and Sumpter, 2001). Sophisticated techniques such as ozonation, membrane filtration and activated carbon have been identified as efficient treatment processes for the elimination of polar environmental pollutants in sewage treatment plants (Ternes et al., 2002).

Furthermore, ozonation has been reported to be a useful method for the reduction of the toxicity of waste water from sewage treatment plants (Gagne et al., 2007). In the present study, the results are in accordance with previous findings indicating that ozone treatment (A5 and A6) is an efficient treatment method. This was reflected both in the results of the chemical analyses and in the zebrafish reproduction and fish sexual development tests (FSDT). A commonly used standard treatment technique in Swedish sewage treatment plants (STPs), such as A3 (Outlet1) corresponds to the treatment line of the main central STP in Stockholm and it appears to be insufficient for eliminating pharmaceuticals or estrogenic substances as observed in the results of the study and the chemical analyses data. Therefore, since aquatic organisms are continually exposed to effluents from several sewage treatment plants, their reproductive capabilities could be adversely affected.

The period of 20 to 60 days post hatch in the zebrafish has been identified as a very sensitive period during which exposure to endocrine disrupting chemicals (EDCs) is likely to elicit effects on sensitive endpoints such as vitellogenin induction and sex ratios (Petersen et al., 2001). In the present study, vitellogenin production was significantly induced in males exposed to sewage effluents in groups A2 (after sedimentation treatment), A3 (outlet 1) and A4 (biofilter) in the fish sexual development test (FSDT). This observation is most likely due to the presence of estrogenic substances in the effluent discharge, which have been reported to be capable of inducing vitellogenin (Vtg) production in fish (Kang et al.,2002a; Van der Belt et al 2003; Ishibashi et al., 2004). In zebrafish, the lowest concentrations of 17α ethinylestradiol (EE2) for inducing Vtg production have been reported at 2-3 ng/L (Örn et al. 2003; Fenske et al. 2001). Although the individual concentration of each estrogenic substance present in the sewage effluents studied is below the level for inducing Vtg, the additive effects of the estrogens might produce the induction.

Moreover, in zebrafish exposed to 17α- ethinylestradiol (EE2) a dose-dependent induction of vitellogenin was observed from 2 ng/L while alterations in the sex

ratios in the direction of females was observed from 1 ng/L (Örn et al., 2003), indicating that sex ratio as an endpoint might be more sensitive than Vtg induction. In the present study, however, there were no significant changes in the sex ratios despite elevated Vtg levels in some groups (A2, A3 and A4).

The only effect observed on gonad development of fish exposed to the effluents was that of the females exposed to water from Line 2 (group A7), which were more immature than females in the other exposed groups. The exposure of fish to estrogens has in many studies been demonstrated to negatively affect gonad maturation, with reductions of mature oocytes, undifferentiated gonads and atresia (Hill & Janz 2003; Van den Belt et al. 2003; Van der Ven et al. 2003; Maack & Segner 2004). Moreover, several undesirable effects on the reproduction of fish exposed to estrogenic substances have been reported, such as delayed or inhibited onset of spawning, reduced number of spawning females, reduced egg production, as well as reductions in the fertilisation and hatchability of the eggs (Hill & Janz 2003; Van den Belt et al. 2003; Maack & Segner 2004). Therefore, the effects observed on reproduction in the current study might be partially due to the presence of estrogens in the effluents.

In recent times, human pharmaceuticals have received increasing attention from environmental and health agencies due to their environmental occurrence and inherent biological activity. In addition, pharmaceuticals in the environment are thought to pose only a low risk for acute toxicity (Fent et al., 2006) and the acute toxicity of pharmaceuticals to aquatic organisms is relatively low (Henschel et al., 1997; Cleuvers, 2003). However, the acute toxicity of pharmaceuticals to aquatic organisms would rarely be observed at measured environmental concentrations since the acute effects detected are usually at concentrations 100-1000 times higher than the residues that occur in the aquatic system (Fent, 2006). Consequently, acute toxicity is pertinent in cases of spills or flooding of sewage treatment plants. This could be a reason why in the fish embryotoxicity test conducted on pharmaceuticals, no adverse effects were observed in the zebrafish embryos at the exposure concentrations ranging from 1µg/L–10mg/L except for clozapine, in which a decrease in the heart rate was observed at the highest concentration. The chorion of the zebrafish has been identified as a barrier against the uptake of xenobiotics in the environment (Braunbeck et al., 2005). This could also account for the absence of noticeable effects in the zebrafish embryos exposed to the various pharmaceuticals. Furthermore, the fish embryotoxicity test (FET) conducted on the pharmaceuticals in this study was less sensitive compared with previous findings on other types of chemicals (Carlsson, 2007; Ali, 2007). However a lack of observed effects in the study conducted might be due to the photodegradation of the pharmaceuticals in the exposure solutions in the laboratory and the differences in the target molecules, receptors and enzyme systems between humans and aquatic organisms such as the zebrafish (*Danio rerio*), since the drugs studied are mostly used in humans.

In a fish embryotoxicity test conducted on zebrafish embryos exposed to six pharmaceuticals, only two of the drugs, gemfibrozil and naproxen produced significant effects. Gemfibrozil elicited an increase in the heart rate at 1mg/L

while naproxen produced a delay in the hatching time in the embryos although it elicited an unclear set of results in the different concentrations used (0.00032 mg/L –1mg/L) (Lundstrom, 2007). This study further confirms that the fish embryotoxicity test might not be sensitive enough for the toxicity testing of pharmaceuticals.

# Conclusions

The results obtained in the study on sewage effluents indicates that ozonation in combination with biofilter is an effective treatment technique. In addition, the treatment processes in Swedish sewage treatment plants need to be optimized in order to prevent undesirable effects on the reproduction of aquatic organisms. Furthermore, since most of the pharmaceuticals tested did not produce observable effects, the fish embryotoxicity test could be improved upon through the addition of more endpoints and the daily renewal of exposure solutions. These could render the test more sensitive for the assessment of the toxicity of environmental pollutants especially pharmaceuticals.
Also, chronic toxicity tests could be conducted in further investigations in order to identify the potential hazards of the drugs since such tests usually span the entire life cycle of the test organisms.

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