Selective serotonin re-uptake inhibitors (SSRIs) are a group of antidepressants that are, due to their efficacy and mild side effects, a first-line treatment against a range of psychological illnesses. However, they are also emerging pollutants and commonly found in effluents from sewage treatment plants and in surface waters in many parts of the world. The prime neurological target of SSRI, the serotonergic system, is not unique to mankind but is virtually identical throughout the vertebrate clade, making many animals susceptible to SSRI effects. The serotonergic system is involved in mood, a wide range of behaviours and various processes during embryonic development and it is therefore suspected that SSRIs may alter the behavioural profile of exposed animals.

This thesis explores the effects of SSRIs on various aspects of fish behaviour, from effects on boldness and feeding behaviour via effects of pharmaceutical cocktails to effects of developmental exposure.

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Selective Serotonin Re-uptake Inhibitors in the Environment
Selective Serotonin Re-uptake Inhibitors in the Environment

Effects of Citalopram on Fish Behaviour

Martin Kellner
Abstract
Selective serotonin re-uptake inhibitors (SSRIs) are a class of psychotropic, anxiolytic and anti-depressant drugs. SSRIs exert their effect by blocking the serotonin re-uptake transporter, causing elevated levels of serotonin in the synaptic cleft that in turn reduces the activity of the stress axis. Due to their safety and efficacy SSRIs are used as a first-line treatment against mental illnesses such as depression and obsessive-compulsive disorder. SSRIs act on the evolutionarily ancient serotonergic system which is virtually identical throughout the vertebrate phylum. Serotonin is involved in an extremely wide range of processes ranging from neuronal and craniofacial embryonic development to regulation of behaviour. Much of this behavioural regulation is mediated by the stress axis and the reproductive neuroendocrine axis.

For classical ecotoxicological measures such as LC50 or reproductive parameters, the lowest observed effect concentrations (LOECs) of SSRIs are substantially higher than concentrations that are found in natural waters. However, suspicions have been rising that SSRIs can exert subtle effects on behaviour at much lower concentrations. Their lipophilic properties and their poor degradation in sewage treatment plants have further reinforced the suspicions that SSRIs may have effects on the behaviour of wild aquatic animals and that such behavioural effects can in turn have ecological repercussions. Using the three-spined stickleback (Gasterosteus aculeatus) and zebrafish (Danio rerio) as model organisms, this thesis focuses on the behavioural effects of SSRIs in fish. The SSRI used throughout this thesis is citalopram, which has been found in fish in coastal areas of the Baltic Sea and other parts of the world.

Effects on behaviour were investigated using several different tests. The novel tank diving test and the scototaxis test measure the fish’s response to a stressful situation by quantifying the propensity to stay close to the bottom and in dark surroundings, respectively. Aggression was measured by lowering a mirror into the fish home tank and counting the number of attacks against the mirror image. Feeding behaviour was measured by counting the number of strikes that the fish performed against a piece of frozen bloodworms. Anxiolytic effects of 15 μg/l and 1.5 μg/l were investigated as well as effects of 0.15 μg/l and 1.5 μg/l on feeding behaviour. Because serotonin is involved in the development of the nervous system, the effects of developmental exposure to 1.5 μg/l was studied after 100 days of remediation. Finally, because SSRIs rarely occur alone in natural waters, the effects on zebrafish of citalopram in a cocktail scenario, with the anxiogenic compound 17α-ethinyl estradiol (EE2) was also investigated.

Citalopram was found to have anxiolytic effects on the three-spined stickleback at 1.5 μg/l and 15 μg/l. Citalopram also suppressed feeding behaviour within a week of exposure and at concentrations as low as 0.15 μg/l. However, this was also the lowest concentration tested, so a LOEC value is yet to be determined. Developmental exposure to 1.5 μg/l was found to increase aggression and feeding behaviour and to reduce locomotor activity. The changes were persistent and remained in adult fish. In the cocktail scenario, the results were ambiguous. Citalopram in single-
substance exposure had anxiolytic effects on one parameter in the novel tank test at 0.1 μg/l. Citalopram enhanced the anxiogenic effects of EE2 in the novel tank test, but in the scototaxis test citalopram appeared to counteract the effects of EE2. It is concluded that citalopram has the potential to affect behaviour in fish at concentrations that have been found in close proximity of sewage treatment plants, but the lowest level at which an effect is exerted is yet to be found.

**Keywords:** Citalopram, SSRI, stickleback, Baltic, behaviour, fish, feeding, anxiety, boldness, serotonin, development.
**Sammanfattning**

Det serotonerga systemet är i hög grad bevarat genom evolutionen och i stort sett identiskt hos människor och övriga vertebrater. Serotonin är inblandat i ett stort antal kroppsliga funktioner, bland annat stressreaktioner, reglering av födobeteende och aggression. Vidare är serotonin med och reglerar nervsystemets tillväxt under embrionaltutvecklingen. Selektiva serotoninåterupptagshämmare (SSRI) är en grupp antidepressiva och anxiolytiska (lugnande) läkemedel vars användning har ökat snabbt på senare år då de är effektiva och har få allvarliga bieffekter. SSRI verkar på det serotonerga systemet, genom att blockera återupptaget av serotonin i den presynaptiska nervvånden. SSRI har tilldragit sig en viss uppmärksamhet som potentiella miljöhhot då de visats kunna påverka ekologiskt relevanta beteenden hos fisk och andra akvatiska organismer vid relativt låga koncentrationer i miljön samtidigt som de bryts ned dåligt i avloppsreningsverk. Avhandlingen fokuserar på ekologiskt relevanta beteende-effekter av SSRI på fisk, med storspigg (*Gasterosteus aculeatus*) och zebrafisk (*Danio rerio*) som modellorganismer. Storspigg är en ekologiskt viktig art i Östersjön och i många andra marina och akvatiska miljöer på norra halvklotet. Zebrafisk är en ofta använd art i forskningskontext med väl kända bete demönster. Citalopram har använts som försökssubstans då det anses vara den SSRI som har minst antal sideeffekter på till exempel det dopaminerga systemet. Citalopram förekommer i utloppsvatten från reningsverk i alla industrialiserade länder och har även hittats i abborre i Östersjön.

Effekter av exponering för SSRI har kvantifierats med hjälp av olika beteendetest. Skototaxi-test och *novel tank diving test* mäter stressresponsen genom att kvantifiera preferensen för närhet till botten och mörka omgivningar. Ätbeteende har mätts som antal utfall mot en matbit under en given tidsperiod och aggression har mätts genom att räkna antal bett mot en spegel. Anxiolytiska effekter undersöks vid koncentrationer på 15 och 1,5 μg/l. Effekter på ätbeteende undersöks vid 0,15 och 1,5 μg/l. Eftersom serotonin är inblandat i embrionaltutvecklingen testades de beteendemässiga effekterna av exponering för 1,5 μg/l under utvecklingen. Då citalopram sällan förekommer ensamt i miljön testades ett cocktailsenario där zebrafisk samtidigt exponerades för citalopram och den anxiogen (oro-framkallande) substansen 17α-etinylestradiol (EE₂).

Citalopram befanns ha anxiolytisk verkan på storspigg samt undertrycka ät beteendet. Effekter på ätbeteendet uppstod inom en vecka efter exponering och vid den minsta testade dosen vilken var 0,15 μg/l, en koncentration som kan förekomma i förorenade ytvatten. Storspigg som exponerats under embrionaltutvecklingen var mer aggressiva, hade lägre lokomotoraktivitet (dvs de rörde sig inte lika mycket) och gjorde fler utfall mot mat då de testades 100 dagar efter exponeringen avslutats. Samtidig exponering för citalopram och den anxiogen substansen 17α-etinylestradiol (EE₂) gav tvetydiga resultat. Citalopram ensam hade ingen signifikant påverkan på beteendet i detta försök. I skototaxitestet motverkade citalopram den anxiogen effekten av EE₂ medan det förstärkte den anxiogen effekten i *novel tank*. 

Sammanfattningsvis har citalopram effekter på ekologiskt relevanta beteenden hos fisk i koncentrationer som förekommer i ytvatten. Det har också permanenta effekter på beteende om exponeringen sker under embryonalutvecklingen. Dessa resultat gör det sannolikt att citalopram och andra SSRI har ekologiska effekter i påverkade vattendrag.
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This thesis is based on the following papers:


**Contributions of the author to the different manuscripts:**

**Paper I:** Original idea, planning, main responsibility for lab work and fish maintenance, data analysis, manuscript writing.

**Paper II:** Original idea, planning, main responsibility for lab work and fish maintenance, data analysis, manuscript writing.

**Paper III:** Planning, main responsibility for lab work, fish maintenance, data analysis and manuscript writing.

**Paper IV:** Took part in lab work, fish maintenance and writing.
1. Introduction

The Baltic Sea is polluted. Its shores are densely populated and intensely farmed, the catchment area being home to some 85 million people (Sobek et al., 2016). Several major cities sit on rivers that flow into the Baltic Sea, including the cities of Riga and St Petersburg, while some are located directly on the coast, such as the cities of Helsinki and Tallinn. These cities constitute point sources of pollution, while there is also more diffuse pollution coming from precipitation, run-off, and smaller, often single-household sewages. The Baltic Sea is, in evolutionary terms, a very young sea and almost all species are of either marine or freshwater origin. In the brackish water of the Baltic Sea, they are often living under constant osmotic stress (Holste et al., 2009; Riisgård et al., 2014), which sometimes manifests itself as a smaller average size than in their native environment. The Baltic Sea constitutes an extreme environment not just in terms of the low salinity, but also because it is a very cold, shallow sea that is periodically covered in ice. The practical salinity in the Baltic Sea follows a gradient from north to south, being 0-3 in the northernmost Bothnian bay, 6-7 in the Baltic sea proper is 6-7 and about 25 in the Kattegatt. This can be compared to a practical salinity of about 30 in the Atlantic (Sildever et al., 2015). The water retention time in the Baltic Sea is long, around 30 years (Reissmann et al., 2009). This gives particles and particle-bound pollutants plenty of time to sediment and may thus influence the accumulation of pollutants. The relative geographic isolation along with the extreme conditions of the Baltic Sea has resulted in both a low species diversity and a low genetic within-species diversity (Johannesson and André, 2006). Biodiversity is an important determinant for the resilience of an ecosystem (Gunderson, 2000), and low-diversity ecosystems are typically less resilient to disturbances than systems with a high level of diversity.

Apart from the natural stressors experienced by a geographically marginal and extreme ecosystem like the Baltic Sea, the 85 million human inhabitants of the watershed are subjecting this fragile ecosystem to further stress. Some of the anthropogenic stressors are physical, such as fishing, dredging, and hydropower plants in the tributaries that keep fish species from reaching their spawning grounds, while some are chemical. Chemical stressors include excessive release of nutrients or different chemicals that may have direct toxic effects such as tributyltin (TBT) (Filipkowska et al., 2016) and copper (Eklund et al., 2010) from anti-fouling paints, pesticides and polychlorinated biphenyls (Szlinder-Richert et al., 2012), polycyclic aromatic hydrocarbons (PAHs) of industrial origin (Biselli et al., 2005; Kammann et al., 2005), brominated flame retardants (Darnerud, 2003) and mercury (Beldowska and Falkowska, 2016). However, some anthropogenic chemicals, such as pharma-
ceuticals, may have more subtle effects, affecting behaviour rather than classic toxicological parameters like survival or reproduction.

1.1 Pharmaceuticals in the environment

Pharmaceuticals and pharmaceutical residues are an environmental problem that until recently, has received relatively little attention compared to other classes of pollutants such as organochlorine compounds, brominated flame retardants, or dioxine but which has nevertheless been increasingly discussed in recent years. Unlike most environmental pollutants, pharmaceuticals are specifically designed to manipulate biological systems, a characteristic that makes them potentially harmful when they are released into the environment. In sewage treatment plant (STP) effluents in developed countries, there is a vast range of different pharmaceutical substances exerting their various effects on fish and other aquatic organisms. To mention just a few, benzodiazepines have been shown to alter social and feeding behaviour in fish (Brodin et al., 2013), ethinylestradiol (the active substance in contraception pills) has been shown to have concentration-dependent effects on anxiety and shoaling (Reyhanian et al., 2011), and non-steroidal anti-inflammatory drugs (NSAIDs, common anti-inflammatory drugs like diclofenac) are known to interfere with reproduction (Stacey, 1976).

While many pharmaceuticals are readily broken down in the human body or in STPs, other pharmaceuticals and pharmaceutical metabolites pass through STPs unchanged (Wahlberg et al., 2008). Some of these are selective serotonin reuptake inhibitors (SSRI), which are the focus of this thesis and constitute the main body of antidepressant pharmaceuticals used in the world. The use of SSRIs is rising in the Baltic region (Fig 1) as well as in the rest of the world. The most commonly used members of the SSRI group are the pharmaceutical substances sertralin (distributed under the names Zoloft, Asentra, Lustral, Serlift, and Concorz), fluoxetine (Fontex, Fluctin, and Prozac), and citalopram (Cipramil and Celexa). Citalopram, which is the most selective SSRI and which has been used throughout this study is relatively persistent in the environment (Writer et al., 2013a) although it is to some extent degraded by photolysis (Kwon and Armbrust, 2005). Citalopram has a high affinity for sediment particles (Kwon and Armbrust, 2008), and sorption to particles and sedimentation is likely the main route of elimination from the water body. Once in the sediment, it appears to be persistent to degradation and can be found in sediments that are more than a decade old (Lahti and Oikari, 2012). Citalopram has been detected in both littoral Baltic waters and fish (Woldegiorgis, 2011), rivers in the Baltic region (Giebułtowicz and Nałęcz-Jawecki, 2014) and in coastal waters and rivers in other parts of the world (Alygizakis et al., 2016; Writer et al., 2013b). Citalopram has also been detected in low nanogram levels in Stockholm drinking water (Woldegiorgis, 2011).
SSRIs are very potent drugs and the concentration needed to exert an effect is very low. The water concentration needed to reach the human therapeutic dose of 0.01 μg/ml plasma (Schulz and Schmoldt, 2003) in fish has been calculated to be 141 ng/l for citalopram (Fick et al., 2010). However, in reality this is a simplification because many other factors such as particle binding and water pH can influence uptake from water to fish (Styrishave et al., 2011). Many of the behavioural and physiological effects of SSRIs on fish can be assumed to have ecological effects, and it is important that the existence of such substances in the Baltic Sea and their consequences are investigated. The Swedish Medical Products Agency (Läkemedelsverket) states in a report from 2011 (Läkemedelsverket, 2011) that environmental legislation concerning pharmaceuticals is insufficient. If the Baltic Sea and its’ unique environment are to remain a viable sea and a resource for future generations, it is imperative that we take this threat seriously and acquire all the knowledge we can, so that informed decisions can be made regarding environmental legislation.

1.2 The serotonergic system

Serotonergic cells in the central nervous system originate from the raphe region in the brainstem in both mammals (Hornung, 2010) and fish (Lillesaar, 2011). Although minor differences exist, the serotonergic system is remarkably well conserved throughout the vertebrate phylum. The serotonergic system is evolutionarily ancient and the primordial 5-HT receptor is believed to have evolved around 700-800 million years ago (Peroutka and Howell, 1994; Gillette, 2006). Serotonin is involved in a plethora of physiological and behavioural contexts in a wide range of cladistic groups ranging from insects (Falibene et al., 2012) to fish (Lillesaar, 2011).
and mammals. In fish, serotonin has been shown to be involved in the regulation of behaviours such as dominance/subordination where high serotonergic activity is associated with subordination (Winberg and Lepage, 1998; Dahlbom et al., 2012) and stress (Winberg et al., 1992) while high extracellular serotonin levels are associated with a lower level of aggression (Perreault et al., 2003; Bell et al., 2007; Dzieweczynski and Hebert, 2012). Serotonin is also involved in the regulation of feeding behaviour in both fish (De Pedro N et al., 1998; Gaworecki and Klaine, 2008; Mennigen et al., 2009; Hedgespeth et al., 2014) and mammals (Voigt and Fink, 2015) where satiety appears to be mediated primarily by the 5-HT1B and the 5-HT2C receptors in a complimentary fashion (Voigt and Fink, 2015 and references therein).

1.2.1 Serotonin production and metabolism

In fish, the nuclei of the serotonergic cells reside primarily in the superior and inferior raphe regions of the brain (Lillesaar, 2011 and references therein). Synthesis of serotonin takes place in the neurons of the serotonergic system which extend from the two raphe centra and innervate most parts of the brain. Production of serotonin starts with tryptophan which is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). 5-HTP is then further converted by the aromatic L-amino acid decarboxylase enzyme into 5-hydroxytryptamine, also known as 5-HT or serotonin (Fig. 1), (Boyle, 2005). Serotonin is stored in vesicles close to the presynaptic nerve ending. When a serotonergic neuron fires, these vesicles fuse with the cell membrane in the presynaptic nerve ending and serotonin is released into the synaptic cleft where it binds to receptors on the postsynaptic nerve ending. Eventually it is taken back up by the 5-HT-transporter protein (5-HTT, also known as SERT, serotonin re-uptake transporter) into the presynaptic nerve ending where it is either recycled or broken down by monoamine oxidase (MAO). SSRIs exert their effect by blocking the 5-HTT. The degradation of serotonin is a two step process that starts with the conversion into 5-hydroxyindolacetaldehyde by MAO which is followed by conversion of the 5-hydroxyindolacetaldehyde into 5-hydroxyindolacetic acid (5-HIAA) by aldehyde dehydrogenase (Bortolato et al., 2010). Because serotonin levels in the synapse are a function of both release rate and re-uptake rate, the quotient between 5-HIAA and 5-HT is sometimes used as an indicator of serotonergic activity.

1.2.2 Receptors

Knowledge about the serotonergic system in fish regarding regulation, receptors and the physiological functions of the various receptors is still limited, but at least six different receptors for serotonin have been found in different fish organs. These receptors are divided into at least 10 subtypes (Kreke and Dietrich, 2008). The receptor types are named 5-HT1, 5-HT2, etc., while the subtype is denoted by a capital letter. For instance, the 5-HT1A receptor is the 5-HT1 receptor subtype A.
serotonergic receptors except one are coupled to G-proteins through which they exert their various effects. The exception is the 5-HT$_3$ receptor which is a ligand-gated ion channel. Most serotonergic receptors are located on the postsynaptic nerve ending, but one exception exists. The 5-HT$_1$ receptor family can be located on the presynaptic nerve ending where it is believed to inhibit the release of dopamine and serotonin (Pytliak M. et al., 2011) among other functions. In other words, it is involved in a negative feedback loop. Indeed, desensitisation of pre-synaptic 5-HT$_1$ receptors is believed to be one of the modes of action of SSRIs (Briley and Moret, 1993). Several 5-HT receptors are involved in the regulation of the HPA/HPI axis (defined below), the most prominent ones being 5-HT$_{1A}$ and 5-HT$_{2A}$ with 5-HT$_{1A}$ having an activating effect on the stress axis while 5-HT$_{2A}$ appears to have an opposite effect (Zhang, 2004). In mammals, 5-HT$_{1A}$ is the most widely distributed serotonergic receptor. It is expressed in various parts of the central nervous system but also throughout the gastrointestinal tract. It is likely involved in the regulation of anxiety, and 5-HT$_{1A}$ agonists like buspiron are used in the treatment of depression (Pytliak M. et al., 2011). Another receptor of interest for this thesis is the 5-HT$_{2C}$ receptor. This receptor is of interest not only because it mediates a wide range of ecologically significant behaviours such as feeding (Higgins et al., 2011), anxiety (Heisler et al., 2007), and aggression (Tsuji et al., 2005) but also because it is a binding site for fluoxetine (but not for the other SSRIs), where the SSRI acts as an antagonist (Owens et al., 2001). Other serotonergic receptors are involved in the regulation of a wide range of behavioural and physiological variables like vasoconstriction, smooth muscle contraction and sexual behaviour (Pytliak M. et al., 2011). It bears being repeated, however, that most of the research on the serotonergic system is being conducted on rodents, and although the serotonergic system is thought to be highly conserved through vertebrate evolution, not all results are necessarily possible to transcribe directly to fish, which are different from terrestrial vertebrates when it comes to morphology, physiology and life history traits.

1.2.3 The HPA/HPI axis

One of the principal systems for conveying serotonergic transmission to behavioural expressions within the vertebrate clade is the stress axis, known as the hypothalamus-pituitary-adrenal (HPA) axis (Wolf, 2003), or the hypothalamus-pituitary-interrenal (HPI) axis in fish. The HPA axis in mammals and the HPI axis in fish are functional homologues. In mammals, the stress axis is stimulated by corticotropin releasing hormone (CRH) released from the hypothalamus. Serotonergic signalling between the CRH system and the serotonergic system takes place by direct synaptic contact (Liposits et al., 1987). The effect of serotonin on the CRH neurons is mediated by 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors (Zhang, 2004). The 5-HT$_{2C}$ receptor might also have an activating effect on the stress axis (Heisler et al., 2007). After release from the hypothalamus, CRH in turn stimulates the release of adrenocorticotropin hormone (ACTH) from the pituitary. ACTH then induces
release of the stress hormone cortisol from the interrenals (in fish) or adrenals in mammals (Dinan, 1996). In fish, manipulation of the stress axis with SSRI has effects on many forms of stress-related behaviour like predator avoidance (Painter et al., 2009; Barbosa et al., 2012; Barry, 2012) and behaviour in novel environments (Sackerman et al., 2010).

1.2.4 The reproductive neuroendocrine (HPG) axis

Besides its role in modulation of the stress axis, serotonin is also important for the regulation of the reproductive neuroendocrine axis (Toufexis et al., 2014). This axis starts with release of gonadotropin releasing hormone (GnRH) from the hypothalamus. GnRH then acts to stimulate release of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland. These two hormones are transported by the blood to the gonads where they control maturation, steroidogenesis, and sperm or egg release (Goodman, 2004). Serotonin is involved in this pathway by stimulating the release of LH which is important for final sperm maturation and ovulation.

Fig 2: Serotonin production and degradation.
From Kreke & Dietrich.
As in the case of the HPI/HPA axis, this stimulation occurs by direct synaptic contact between serotonergic neurons and GnRH neurons (Kiss and Halász, 1985). There is also evidence of direct interaction between the HPA axis and the HPG axis because gonadal hormones influence the response to stress (Viau and Meaney, 1991; Lund et al., 2004). While most research on the stress and reproductive axis has been conducted on mammals, there is some evidence that these functions are highly conserved between fish and mammals. For example, fluoxetine has been shown to disrupt the reproductive axis in male goldfish (*Carassius auratus*) (Mennigen et al., 2010), and injection of 5-HT into goldfish has been shown to stimulate the release of gonadotropine hormones. The release is inhibited by ketanserin, a 5-HT$_2$ receptor antagonist (Somoza et al., 1988a), which would indicate that the effect is mediated by one or several of the receptor subtypes in this family. The quality of nests built by fluoxetine-exposed stickleback males is lower than that of nests built by unexposed males (Sebire et al., 2015), but it is not clear if this effect is mediated by the HPG axis.

1.3 SSRI

Selective serotonin re-uptake inhibitors are a group of antidepressant pharmaceuticals that exert their effects by blocking the re-uptake of serotonin into the presynaptic nerve terminal which causes elevated levels of serotonin in the synaptic cleft and improved mood in many depressed patients. Citalopram is the SSRI used throughout this thesis. It was chosen because it is generally regarded as the most selective SSRI currently on the market and less likely to introduce effects not related to the serotonergic system (Kitaichi et al., 2010). Citalopram is a racemic mixture of the R- and S-enantiomers (fig 3). The S-enantiomer (also known as escitalopram) is responsible for virtually all of the serotonin re-uptake inhibiting effect (Hyttel et al., 1992). Some studies show that the R-enantiomer has an inhibiting effect on the S-enantiomer, such that the effect of a certain amount of escitalopram does not directly correspond to twice the amount of citalopram which would otherwise have been anticipated. This inhibition probably occurs through binding to an allosteric binding site (Zhong et al., 2009 and references therein). In humans, approximately 12% of the administered dose is excreted unchanged through the urine (Milne and Goa, 2012). The rest is broken down to citalopram-N-oxide, citalopram propionic acid and the active metabolite desmethylcitalopram (Hyttel et al., 1992). Desmethylcitalopram is then further degraded to didesmethylcitalopram (Sangkuhl et al., 2011). Biotransformation of citalopram is mainly due to the activity of different enzymes in the Cytochrome P450 family (Hiemke and Härtter, 2000). Both desmethylcitalopram and didesmethylcitalopram are found in the environment along with the parent compound (Vasskog et al., 2008).
Both the racemic mixture citalopram and the S-enantiomer escitalopram are used in the treatment of depression in humans and are commercially available. On the margin, this may have some significance for the current thesis; Because of the marketing of both citalopram and escitalopram, the ratio between the two enantiomers in the environment might deviate from the 1:1 ratio in citalopram. Hence, in the environment the effect may be greater than experiments with only citalopram will show. While sharing the same main mode of action, other SSRIs than citalopram have slightly different profiles. For example, in addition to their SSRI activity both fluoxetine and sertraline elicit elevated noradrenaline and dopamine levels (Kitaichi et al., 2010), and unlike the other SSRIs, fluoxetine acts as an antagonist of the 5-HT$_{2C}$ receptor (Owens et al., 2001). In mammals, the 5-HT$_{2C}$ receptor is involved in the regulation of a wide range of behaviours, including locomotion, feeding, penile erection, and anxiogenesis (Barnes and Sharp 1999 and references therein). The binding of fluoxetine to the 5-HT$_{2C}$ receptor is quite likely responsible for at least some of the adverse side effects of fluoxetine observed in humans such as sexual dysfunction.
1. INTRODUCTION

1.3.1 Environmental occurrence and degradation

SSRIs are present in many aquatic environments, as far north as outside the town Longyearbyen on Spitsbergen (Vasskog et al., 2008) but most studies seem to have been performed in northern Europe and North America (table1). The near omnipresence of SSRIs in STP effluents and effluent-dominated streams partially reflects their poor degradation rates in STPs. The average degradation of citalopram in a dataset from 15 Swedish STPs was -6% (Wahlberg et al., 2008), indicating that there was on average more citalopram in the STP effluents than in the influents. This is likely because when citalopram leaves the human body, the substance is conjugated but the conjugate is lost due to bacterial activity in the STP. The results from the Swedish studies are echoed in a study from China where citalopram was consequently detected at higher concentrations in effluents than in influents from municipal STPs (Yuan et al., 2013). In some studies, degradation in nature or in semi-natural settings has been measured. The half-life of fluoxetine during microbial degradation under dark conditions at 15°C has been estimated to be between 5.9 and 9.8 days (Benotti and Brownawell, 2009). The stability of citalopram under simulated sunlight in the absence of microorganisms has been found to be pH dependent. Thus, over a 30-day period less than 0.5% of the citalopram was degraded at pH 5 or pH 7, while degradation was faster at a pH 9. The same study
found that in sterilized lake water and in synthetic humic acid, the degradation was faster than in all buffers despite the pH being lower than 9, indicating that the tested natural waters had some photosensitising properties, possibly related to humic acid content (Kwon and Armbrust, 2005). The stability of sertraline has been evaluated in different concentrations in a microcosm setup and it was found that the mean half-life of sertraline was 6.0–6.5 days in the different treatments. There was no difference between autoclaved water and non-autoclaved water, and virtually no degradation occurred in controls kept under dark conditions. Thus, degradation occurred mainly due to photolysis (Lam et al., 2004). In a Danish study also using a microcosm setup, citalopram was found to be degraded primarily via biological pathways while fluoxetine and sertraline were mainly degraded by photolysis. This study also confirmed that all three SSRIs showed strong sorption to sludge and exhibited very slow degradation (Styrishave et al., 2011). Degradation rates and different routes of degradation (biological, photolytic, etc) make little difference in close proximity to the pollution source (e.g. an STP effluent) but can determine how far from the source the pollutants will spread and exert an effect before they are degraded. From the studies described above, it can be concluded that pharmaceuticals within the SSRI group are degraded by different means and that several different factors play a role in their elimination from the water body, i.e. pH, particle sorption, susceptibility to biological degradation and light conditions. In addition, temperature should be mentioned since this variable may have an effect on the biological degradation rate.

Table 1: 18 measured STP effluent concentrations (MEC) of citalopram in 15 studies and their risk quotient (RQ) when the LOEC 150 ng/l from this thesis is employed. No safety factor or compensation for dilution has been used.

<table>
<thead>
<tr>
<th>MEC (ng/l)</th>
<th>LOEC (ng/l)</th>
<th>RQ (MEC/LOEC)</th>
<th>Country</th>
<th>Study</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>377</td>
<td>100</td>
<td>3.77</td>
<td>Sweden</td>
<td>Helmfrid et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>268</td>
<td>100</td>
<td>2.68</td>
<td>Sweden</td>
<td>Lindberg et al. (2014)</td>
<td></td>
</tr>
<tr>
<td>33.8</td>
<td>100</td>
<td>0.338</td>
<td>EU</td>
<td>Loos et al. (2013)</td>
<td>Mean from 90 STPs in the EU</td>
</tr>
<tr>
<td>203</td>
<td>100</td>
<td>2.0</td>
<td>Canada</td>
<td>Metcalfe et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>100</td>
<td>1.80</td>
<td>Germany</td>
<td>Schlüsiens et al. (2015)</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>100</td>
<td>2.00</td>
<td>Germany</td>
<td>Schlüsiens et al. (2015)</td>
<td></td>
</tr>
<tr>
<td>9.2-382</td>
<td>100</td>
<td>0.92-3.82</td>
<td>Norway</td>
<td>Vasskog et al. (2006)</td>
<td>Samples from several STPs</td>
</tr>
<tr>
<td>&lt;LOQ-238</td>
<td>100</td>
<td>0-2.38</td>
<td>Norway</td>
<td>Vasskog et al. (2008)</td>
<td>Samples from several STPs</td>
</tr>
<tr>
<td>86</td>
<td>100</td>
<td>0.86</td>
<td>Sweden</td>
<td>Woldengjøv et al. (2006)</td>
<td>Average of 17 samples from 15 STPs</td>
</tr>
<tr>
<td>&lt;10-520</td>
<td>100</td>
<td>0-5.2</td>
<td>USA</td>
<td>Winter et al. (2013)</td>
<td>24 different STPs in Minnesota</td>
</tr>
<tr>
<td>2-162</td>
<td>100</td>
<td>0.02-1.62</td>
<td>China</td>
<td>Yuan et al. (2013)</td>
<td>1 psychiatric hospital treatment plants and 3 municipal treatment plants</td>
</tr>
<tr>
<td>&lt;LOD-95.6</td>
<td>100</td>
<td>0-0.956</td>
<td>Portugal</td>
<td>Silva et al. (2014)</td>
<td>From 8 different Portuguese STPs</td>
</tr>
<tr>
<td>260</td>
<td>100</td>
<td>2.60</td>
<td>Sweden</td>
<td>Griebelova et al. (2014)</td>
<td>Mean 14 samples from one STP</td>
</tr>
<tr>
<td>84</td>
<td>100</td>
<td>0.84</td>
<td>Slovakia</td>
<td>Mackulak et al. (2015)</td>
<td></td>
</tr>
<tr>
<td>165-303</td>
<td>100</td>
<td>1.65-3.03</td>
<td>Germany</td>
<td>Gurtke et al. (2015)</td>
<td></td>
</tr>
<tr>
<td>122-49.2</td>
<td>100</td>
<td>0.122-0.692</td>
<td>Iceland</td>
<td>Huber et al. (2016)</td>
<td></td>
</tr>
<tr>
<td>101-540</td>
<td>100</td>
<td>1.01-5.4</td>
<td>Faroe islands</td>
<td>Huber et al. (2016)</td>
<td></td>
</tr>
<tr>
<td>130-102</td>
<td>100</td>
<td>1.3-1.92</td>
<td>Greenland</td>
<td>Huber et al. (2016)</td>
<td></td>
</tr>
</tbody>
</table>
In Sweden, citalopram is the most commonly prescribed SSRI, which is also reflected in the concentrations of the various substances in STP effluents. In 2006, over 1,700 kg of citalopram was sold in Sweden, while the amount of fluoxetine sold was less than 500 kg. Between 2006 and 2009, this resulted in an annual calculated release of 34 kg of citalopram into the Baltic Sea from the Stockholm area alone. Typical levels of citalopram in STP effluents were 0.1-0.25 μg/l. Levels of sertraline and fluoxetine were lower, around 0.008-0.035 μg/l (Woldegiorgis, 2011). In another study conducted in the Swedish county of Östergötland the mean concentration of citalopram in sewage treatment plant effluents was 0.38 μg/l (Helmfrid et al., 2010). In the SSRI group, the different drugs have different properties regarding their tendency to bind to particles. This is evident in the study from Östergötland, where sertraline and fluoxetine were not discovered in the effluents from all STPs and thus were omitted from further analysis. The average effluent concentration of citalopram was, as previously mentioned, 0.38 μg/l. In the same study, citalopram was detected in a range between 0 and 760 μg/kg in sludge. Sertraline was present in sludge in a concentration range between 240 and 1,200 μg/kg, and fluoxetine was detected at concentrations between 5 and 210 μg/kg. Results from a study performed in the US where both sediment and water data are presented are similar with regard to water-sediment distribution (Schultz et al., 2010). Different affinities for particle binding has ecological relevance because it might influence where in the environment the substances eventually end up and what organism groups are exposed. Because SSRIs are a diverse group of pharmaceuticals, the total concentration of SSRIs can be quite high even if the concentration of each substance is low (Mennigen et al., 2011), and the effects on fish and other aquatic organisms of such mixtures are hard to predict given that different SSRIs have slightly different effects (Bymaster et al., 2002) despite their common primary mode of action. As an example, in one measurement from the Vestfjorden Avløpsselskap STP close to Oslo, Norway, the level of the most frequent parent compound (citalopram) was 238.4 ng/l, but the total SSRI content was 561.8 ng/l, including the active metabolites desmethylcitalopram and norfluoxetine (Vasskog et al., 2008).

1.3.2 SSRI occurrence in aquatic biota

The log K_{ow} value of citalopram is 1.39, which is the highest among the SSRI (Kwon and Armbrust, 2008). However, all SSRIs are weak bases and will therefore occur as charged molecules at lower pH. Therefore, the log K_{ow} as measured in an unbuffered solution is a simplification because SSRIs will be less charged and more soluble in fat under those conditions than under environmental pH. Increased toxicity with higher pH has been demonstrated for both fluoxetine (Nakamura et al., 2008) and sertraline (Valenti et al., 2009), and this is almost certainly true for citalopram as well. Nakamura et al. (2008) also demonstrated that for fluoxetine, bio-availability is
positively correlated to pH, pointing to increased uptake as a likely reason for the increased toxicity. It is known that both fluoxetine and its active metabolite norfluoxetine are being bioaccumulated in fish, especially in the brain (Brooks et al., 2005; Schultz et al., 2010). In addition to citalopram, other SSRIs such as fluoxetine, paroxetine and sertraline are also present in the environment (Brooks et al., 2005; Vasskog et al., 2008; Schultz et al., 2010; Woldegiorgis, 2011). Citalopram has been found in perch liver from Riddarfjärden in the inner parts of the Stockholm archipelago at a concentration of 0.3-0.5 ng/g wet weight (Woldegiorgis et al., 2006). Since the concentrations of citalopram in surface water in this area are typically a few nanograms per litre, this could be an indication that citalopram bioaccumulates in fish. In another study, a citalopram concentration range of 0-60 ng/l in water yielded a brain concentration of 0-0.07 ng/g in the teleost species white sucker (*Catostomus commersonii*), which means that the concentration in the brain was approximately the same as in the surrounding water. In the same study, the concentration ranges for fluoxetine were 0-9 ng/l and 0-0.6 ng/g in water and brain tissue, respectively. For sertraline the concentrations were 2-4 ng/l and 0-1.8 ng/g. Lastly, the corresponding figures for paroxetine were 0-4 ng/l and 0-0.02 ng/g (Schultz et al., 2010). Thus, all SSRIs for which the samples were analysed were found in brain tissue. In addition, the active metabolite norfluoxetine and the more or less inactive metabolite norsertraline were found. In a study by Brooks (2005), both sertraline and fluoxetine were found in the brains and other tissues of wild channel catfish (*Ictalurus punctatus*), but unfortunately citalopram was not analysed in this study. The mean concentrations of fluoxetine and setraline in the brains in that study were 1.58 ng/g and 4.27 ng/g, respectively.

### 1.3.3 Physiological effects of SSRI exposure on fish

Citalopram and other SSRIs have many physiological effects on fish, some of which may have ecological consequences. The most studied effects of SSRI concern the reproductive axis, and appear to differ between fish species and/or dose. Fluoxetine dissolved in water elevates the risk of embryonic abnormalities in Japanese medaka (*Oryzias latipes*) in a range between 0.1 and 5.0 μg/l, and it increases the concentration of estradiol in plasma at the lowest concentration only, indicating that the dose-response curve might not be linear (Foran et al., 2004). Fluoxetine at a concentration of 32 μg/l reduces the levels of ovarian 17β-estradiol and the clutch size in zebrafish. This concentration also reduces the ovarian expression of the LH receptor and the FSH receptor which might cause the reduced clutch size (Lister et al., 2009). In goldfish, fluoxetine injected at a dose of 5 μg/g b.w. reduces plasma estradiol and expression of oestrogen receptors in the hypothalamus and telencephalon as well as the expression of isotocin, a neuropeptide that stimulates reproductive behaviour in fish (Mennigen et al., 2008). Fluoxetine has been reported to potentiate the stimulatory effect of 5-HT on serum gonadotropin levels in goldfish of both sexes (Somoza et al., 1988b). Pharmacokinetically, SSRIs have an in-
hibiting effect on cytochrome P450 enzymes and may interfere with the degradation of other drugs that a fish is exposed to (Park et al., 1995; Hiemke and Härtter, 2000). One frequently reported effect of SSRIs on fish is reduced levels of the stress hormone cortisol (Lepage et al., 2005), which is an effect of the influence of serotonin on the HPI axis (see section 1.3).

1.3.4 Behavioural effects of SSRI exposure in fish

In the three-spined stickleback and probably in other species as well, different challenges elicit different neuroendocrine responses (Bell et al., 2007). Neuroendocrine responses are closely linked to behaviour. For example, aggression against a conspecific and predator inspection are positively correlated to noradrenalin levels, while serotonin levels are positively correlated to predator inspection but negatively correlated with conspecific aggression (Bell et al., 2007). SSRI exposure affects several genes involved in stress response (Wong et al., 2013). Behavioural effects that are mediated by the HPI axis (i.e. anxiolysis) belong to the most intensely studied effects of SSRIs on fish. They are important from an ecological perspective because fish that are less anxious may be easier prey. Tests used to study such behavioural effects often involve exposure to a novel environment such as in the novel tank test (Egan et al., 2009; Sackerman et al., 2010; Wong et al., 2010; Olsén et al., 2014) or scototaxis (Maximino et al., 2011) but can also be exposure to other stressors such as conspecific alarm substances (Barbosa et al., 2012; Barry, 2012).

The effects of serotonin on the stress axis give rise to a number of behavioural changes. Sackerman et al. (2010) found a significant anxiolytic effect on zebrafish behaviour in the novel tank test after a 3 minute exposure to 100 mg/l of citalopram, Wong et al. (2010) found significant anxiolytic effects of 2 weeks exposure to 100 μg/l of fluoxetine on zebrafish novel tank behaviour, Kellner et al. (2016) found significant anxiolytic effects of 3 weeks exposure to 1.5 and 15 μg/l citalopram on three-spined stickleback behaviour, and Olsén et al. (2014) found significant effects in the novel tank test after 3 weeks of exposure to 2.3 and 15 μg/l of citalopram. Valenti et al. (2012) showed that sertraline decreases shelter-seeking behaviour in the fathead minnow (*Pimephales promelas*). In a study on latency to the initiation of escape behaviour and escape velocity among larval fathead minnows, it was found that the total escape response was impaired by venlafaxine and fluoxetine, but not by sertraline, when the larvae were exposed as embryos (Painter et al., 2009). It is not clear, however, if this is an anxiolytic effect or if it is due to effects on locomotor behaviour. Effects of SSRI exposure on feeding behaviour are frequently reported in the literature. Gaworecki & Klaine (2008) found that hybrid striped bass (*Morone saxatilis* × *M. chrysops*) exposed to ambient fluoxetine exhibited an increased, dose-dependent latency to catch prey, Kellner et al. (2014) found that 1.5 μg/l and 0.15 μg/l citalopram partially inhibited feeding in the three-spined stickleback, and Mennigen et al. (2009) showed that female goldfish injected with fluoxetine showed a reduced appetite and reduced weight gain. Another important behavioural variab-
le that appears to be influenced by SSRI exposure is aggression, which is often reduced after SSRI exposure (Perreault et al., 2003; Lepage et al., 2005). One theory as to the reason for the reduced aggression is that SSRIs affect the arginine vasotocin/vasopressin (AVT/AVP) system (Semsar et al., 2004). Because high serotonergic system activity is symptomatic of fish in a subordinate position (Dahlbom et al., 2012), it has been hypothesised that social hierarchies among fish may be reversed as an effect of SSRI exposure, and there is some experimental evidence to support this theory (Lepage et al., 2005). This could lead to a weaker natural selection for strong individuals and hence have a negative effect on the species’ gene pool.

1.4 Environmental risk assessment

Table 1: Lowest Observed Effect Concentration (LOEC), No Observed Effect Concentration (NOEC) for different endpoints and organisms in previous ecotoxicological studies of Citalopram. Time frames longer than 72 h have been classified as chronic.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Time frame</th>
<th>LOEC</th>
<th>NOEC</th>
<th>Organism</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproduction</td>
<td>Chronic</td>
<td>4 mg/l</td>
<td>0.8 mg/l</td>
<td>Ceriodaphnia dubia (zooplankton)</td>
<td>Henry et al (2004)</td>
</tr>
<tr>
<td>Mortality (7-8 days)</td>
<td>Chronic</td>
<td>4 mg/l</td>
<td>0.8 mg/l</td>
<td>Ceriodaphnia dubia (zooplankton)</td>
<td>Henry et al (2004)</td>
</tr>
<tr>
<td>Aggression</td>
<td>Chronic</td>
<td></td>
<td>100 µg/l</td>
<td>Rainbow trout fry</td>
<td>Holmberg et al (2010)</td>
</tr>
<tr>
<td>Sexual behaviour</td>
<td>Acute</td>
<td></td>
<td>100 µg/l</td>
<td>Guppy</td>
<td>Holmberg et al (2010)</td>
</tr>
<tr>
<td>Foot detachment</td>
<td>Acute</td>
<td>405 pg/l</td>
<td></td>
<td>Leptoxia carinata (freshwater snail)</td>
<td>Fong &amp; Hoy (2012)</td>
</tr>
<tr>
<td>Foot detachment</td>
<td>Acute</td>
<td>4.05 µg/l</td>
<td></td>
<td>Stagnicola elodes (freshwater snail)</td>
<td>Fong &amp; Hoy (2012)</td>
</tr>
<tr>
<td>Sexual behaviour</td>
<td>Chronic</td>
<td></td>
<td></td>
<td>Guppy</td>
<td>Olsen et al (2014)</td>
</tr>
<tr>
<td>Novel tank behaviour</td>
<td>Chronic</td>
<td>15 µg/l</td>
<td>2.3 µg/l</td>
<td>Guppy</td>
<td>Olsen et al (2014)</td>
</tr>
<tr>
<td>Freezing</td>
<td>Chronic</td>
<td>2.3 µg/l</td>
<td>0.2 µg/l</td>
<td>Guppy</td>
<td>Olsen et al (2014)</td>
</tr>
<tr>
<td>Shoaling</td>
<td>Chronic</td>
<td></td>
<td>15 µg/l</td>
<td>Guppy</td>
<td>Olsen et al (2014)</td>
</tr>
<tr>
<td>Mortality (3 days)</td>
<td>Acute</td>
<td>10 mg/l</td>
<td>1 mg/l</td>
<td>Japanese medaka larvae</td>
<td>Chiffre et al (2016)</td>
</tr>
</tbody>
</table>

Previously reported LOEC and NOEC values for various organisms and endpoints are summarised in table 1. Environmental risk assessments of chemicals are typically based on toxicity tests measuring LC50 (the concentration in water that kills 50% of the experimental animals over a given period of time) or tests that measure the production of offspring. Frequently, toxicity is divided into acute (often 48 h) and chronic (7-14 days). Thus, the 48-hour LC50 for citalopram on the zooplankton Ceriodaphnia dubia is 3.90 mg/l, while the chronic (8-days) LOEC for a reproductive endpoint is 4 mg/l (Henry et al., 2004). Similarly, the 72-hour survival of Japanese medaka (Oryzias latipes) larvae is 38.9% in a solution of 10 mg/l citalopram (Chiffre et al., 2016). Chiffre et al. (2016) also found increased larval thigmotaxis in the lowest tested concentration of citalopram, 10 µg/l. LOECs can also be predicted by models, such as Quantitative Structure-Activity Relationship (QSAR) models. Using this approach, it has been noted that while the reported predicted no effect concentration (PNEC) from literature and using a safety factor of 1000 was 4.9 µg/l, the PNEC using the QSAR model with a safety factor of only 50 was 0.004 µg/l (Ahlford, 2007). In recent years however, it has become more and more
apparent that for psychoactive substances such as SSRIs, simple endpoints like LC50 or reproductive endpoints may not be enough. The reason for this is mainly that the behavioural effects of psychoactive pharmaceuticals occur at concentrations far below the level at which a pharmaceutical becomes acutely toxic. In fact, a wide gap between the therapeutic dose and toxic levels is considered a virtue in pharmaceutical development since it reduces the risk of accidental overdoses.
2. Aims of this thesis

The overall aim of this project was to investigate the behavioural effects of SSRI exposure in fish. In detail, the aims were:

1. To investigate the effects of citalopram on fish feeding behaviour (three-spined stickleback, Paper I).
2. To quantify the latency to onset of effect on feeding behaviour and to find a lowest observed effective concentration, LOEC (three-spined stickleback, Paper I).
3. To investigate the anxiolytic effects of citalopram in fish (three-spined stickleback, Paper II).
4. To investigate the effects of developmental exposure to citalopram in fish (three-spined stickleback, Paper III).
5. To test the combined effects of the anxiogenic compound 17α-ethinyl estradiol and citalopram on stress-related behaviour in fish (zebrafish, Paper IV).
3. Methods

3.1 The three-spined stickleback

The three-spined stickleback is used in all studies in this thesis except Paper IV. The three-spined stickleback is a small fish, up to 10 cm in length, which is present in marine environments in the entire northern hemisphere and in many freshwater lakes. There are also anadromous populations, i.e. populations that migrate between freshwater and marine conditions. The three-spined stickleback is an emerging model species (Ioanna Katsiadaki et al., 2007; Huntingford and Ruiz-Gomez, 2009) and is well characterised regarding both behaviour (Ward et al., 2004) and physiological responses to stress (Pottinger et al., 2002, 2013). In addition, the whole genome has been sequenced, and they are easy to maintain in captivity. For those reasons, it is considered a suitable environmental sentinel species (Pottinger et al., 2002).

In addition to its virtues as an experimental organism, the three-spined stickleback fills an important ecological niche in the Baltic Sea, mostly as prey for larger fish (Bergström et al., 2015) and birds (Lehikoinen et al., 2011). During spawning season the male sticklebacks develop a pronounced nuptial colouring (Fig. 5) and become territorial and very aggressive. They frequently attack each other as well as anything else that is perceived as a threat. Before spawning, the male three-spined sticklebacks build a nest out of algae, sand, or other materials that are locally available in which the female fish lay their eggs. The males then practice paternal care of the young until they have hatched and can provide for themselves. This care-taking involves fanning fresh water into the nest as well as chasing away potential predators. The mating behaviour is complex and involves the male “dancing” in front of the female. In some cases the courting can also be reversed so that the female courts the male. The female has a strong preference for males with a red belly (Künzler and Bakker, 2001), the brighter red the better. Apart from the intricate mating behaviour, the three-spined stickleback also exhibits a range of other behaviours that are susceptible to SSRIs such as aggression, thigmotaxis (the preference for areas close to the wall of an aquarium or other objects), scototaxis (light/dark preference), shoaling, etc. The stickleback’s near omnipresence in Swedish waters along with their complex behaviour, their general robustness, and their status as an emerging model organism in various fields of research make them an almost ideal organism for behavioural studies on fish.
3.2 Zebrafish

The zebrafish (Danio rerio) is a small cyprinid fish, originating from India and Bangladesh and a long established model species with a well characterised behaviour. While not quite as robust as the three-spine stickleback, it is easy to keep in the laboratory and the generation time is short, around 3-4 months, which allows for high throughput at a low cost. Like the three-spined stickleback, the zebrafish displays a predictable response to stress in the scototaxis (Maximino et al., 2010) and the novel tank diving tests (Egan et al., 2009). The zebrafish is a shoaling species, and like the scototaxis and bottom dwelling preference, its shoaling behaviour is sensitive to anxiogenic (Hallgren et al., 2011) and anxiolytic (Gebauer et al., 2011) effects. Fertilization in the zebrafish is external and most organs are developed within 36 hour (Kimmel et al., 1995) which has made the zebrafish popular in within the field of developmental biology. The whole genome is sequenced which adds to the popularity of the zebrafish and there are thousands of well defined genetic lines to choose from (Geisler et al., 2016).

3.3 Fish sources and maintenance

The three-spined sticklebacks used in the studies underlying this thesis were wild-caught in Skåne in southern Sweden using a net, except the fish used in Paper III which were bred from wild sticklebacks caught on the Swedish east coast and raised in the stickleback facility at Stockholm University. Fish were handled carefully and allowed at least one week to acclimatise before any experiment took place. Salinity at the location of capture is approximately 0.7% (Sildever et al., 2015), but during acclimatisation salinity was gradually reduced to tap water levels. Sticklebacks caught in the wild were transported to the stickleback facility at Södertörn University within 48 hours. Once acclimatisation to the conditions in the facility was over, the sticklebacks were fed bloodworms daily to satiation and were kept either in a flow-through system or, during experiments, under a semi-static water regime where half the water was changed with room temperature, aerated water every second day. The tanks were cleaned from faeces and food residues at the same time. Sticklebacks were kept in a 8:16 day/night cycle to keep them from sexual maturation, which might have influenced the results. Zebrafish of the wild type strain AB were obtained from the Karolinska Institute Zebrafish Core Facility and kept under standardized conditions (25-27°C, pH 7.8, conductivity 20.7 mSi) and fed three times daily with Sera dry flakes (Vipan, Germany) and Artemia nauplii (Artemia International LLC, USA). Zebrafish were kept in a 12:12 day/night cycle. During the experiment with zebrafish in Paper IV, the water regime was semi-static with half the water volume being changed daily. In Paper II, euthanasia was performed using phenoxyethanol. In the other studies, MS-222 was used. All handling and keeping of fish was compliant with Swedish law and was permitted by the ethical committee on
animal experiments in southern Stockholm (dnr 61-13 and S28-15) or northern Stockholm (dnr N 22-15).

3.4 Exposure to SSRIs

Racemic Citalopram (1:1 mixture of the R- and S-enantiomeres) is used in Paper I–IV in this thesis. Nominal concentrations in the aquaria were obtained by preparing a stock solution from Milli-Q water that was mixed into the aquaria in the correct proportions. In Paper IV, EE2 was used in addition to citalopram. EE2 solutions were made from a stock solution of EE2 dissolved in acetone. Thus, all final working solutions including the single-substance citalopram solutions contained 10 ppm acetone. The concentration of citalopram in the aquaria was measured using HPLC-MS in the same way in Paper I and II. See either paper for details.

3.5 Behavioural endpoints

Anxiety and response to novelty

Small fish are typically prey for both bigger fish and piscivorous birds. In other words, natural selection (Darwin, 1859) will favour those individuals whose behaviour protects them from such threats. For small fish, such as the three-spined stickleback, the best survival strategy is often to hide, which is easiest to do in dark environments and close to the bottom where the fish cannot easily be seen in silhouette against the sky. As a result of those selective pressures, three-spine sticklebacks and many other small fish have developed a propensity to stay in dark conditions (Maximino et al., 2010) and close to the bottom (Levin et al., 2007) when stressed or anxious. Such behavioural patterns can be exploited to quantify anxiety and boldness in fish through different behavioural tests where fish are subjected to stress. One such behavioural test is the novel tank test (Egan et al., 2009). In the novel tank test, fish are dropped into an aquarium that is unknown to them which causes stress. The test aquarium has a horizontal line drawn on the glass approximately half-way between the bottom of the aquarium and the surface and anxiety is quantified by measuring the latency before the fish first crosses the horizontal line, the number of times that it does so during a certain time period and the total time spent in the upper half of the aquarium. More anxious fish will then exhibit a longer latency time, fewer transitions to the upper half and spend more time there. Conversely, because SSRIs are anxiolytics, SSRI-exposed fish are expected to exhibit a shorter latency, make more transitions to the upper half and spend more time there. The novel tank test is employed in Papers II, III and IV in this thesis. Some fish exhibit a behaviour known as freezing. Freezing is when the fish lies motionless in the water, often just above the bottom and is likely a way to reduce the risk of discovery by a predator. Freezing is sometimes included in the novel tank test, as in Paper II in this thesis, although it was not part of the original design. In all
three papers in this thesis where the novel tank test has been employed, locomotor activity was quantified as a part of the novel tank test by superimposing a grid over the video screen and counting the number of times that the fish crossed any vertical or horizontal line in the grid.

The propensity of stressed or anxious organisms to stay over dark bottoms is known as light/dark preference or scototaxis. This behaviour too, can be exploited as a biomarker for effects on the HPI axis, in the form of a scototaxis test of anxiety, where one half of the aquarium has white walls and bottom while the other half is black (see materials and methods sections in Papers III and IV). Analogous to the novel tank test, the latency to the first cross to the white half of the aquarium, the number of crosses to the white half, and the total time spent in the white half of the aquarium can be used as measures of stress. In Paper II, a novel object test was employed to measure exploratory behaviour when confronted with a previously unknown object. The object was lowered in the middle of three concentric circles drawn on the bottom of the aquarium and exploratory behaviour was measured as the latency to enter into each circle, the number of entries, and the time spent in each circle.

**Feeding behaviour**

Feeding behaviour is sensitive to manipulation by SSRI. In Papers I and III, feeding behaviour was measured by counting the number of attacks against a piece of food (frozen bloodworms) in the fish home tank over the course of 10 minutes.

**Aggression**

In Paper III, aggressive behaviour was quantified by lowering a mirror into the fish’s home tank and counting the number of bites that the fish performed against the mirror image. While this is not exactly the same as aggression against a real life antagonist, it is likely to produce a good measure of aggression (Elwood et al., 2014).

### 3.6 Statistics

All statistical analysis were performed in R (R core team, 2015) running under Ubuntu Linux (Canonical Group Ltd, London, UK) or, in Paper IV, under Windows (Microsoft Inc., Redmond, USA). Most of the statistical analyses in this thesis are linear mixed models or generalised linear mixed models using the lme4 (Bates et al., 2015) and multcomp (Hothorn et al., 2008) packages. For count data like bites and crossings, a Poisson distribution has been assumed for residuals. In models for time and weight data, a Gaussian error distribution has been assumed. When necessary, response variables have been log or square root transformed. Date (Paper I, Paper III), aquarium (Paper I and II) and sibling group (Paper III) have been used as random factors to avoid pseudo replication. In a few cases where it was not possible to obtain a proper distribution of the residuals, a Kruskal-Wallis test was used. Signifi-
cant effects of different explanatory factors were analysed with type II Wald $\chi^2$ tests using the car (Fox and Weisberg, 2011) package. In Paper II the ANOVA was followed by a pairwise comparison of means using Tukey Contrasts. In Paper IV, ANOVA was used followed by Dunnet’s post-hoc test and Tukey Contrasts.
4. Results and discussion

4.1 Feeding behaviour (Paper I)

Paper I discusses the effects of low-dose chronic citalopram exposure on feeding behaviour in the three-spined stickleback. The study consisted of two parts, where the first part was an attempt to determine the latency to onset of effects on feeding behaviour during exposure. Female three-spined sticklebacks were exposed to 0 μg/l or 1.5 μg/l citalopram dissolved in the ambient water for 21 days. Feeding behaviour was recorded on video before onset of exposure and then weekly for three weeks. Reduced feeding behaviour was manifest already after one week and persisted throughout the study. The second part of the study was an attempt to find the minimum concentration in the ambient water that had an effect on feeding behaviour (the LOEC value). In this part of the study, female sticklebacks were exposed to 0 μg/l, 0.15 μg/l and 1.5 μg/l citalopram. Both of the treated groups showed significantly suppressed feeding behaviour compared to control fish but were not significantly different from each other, indicating a flat dose-response curve in this concentration range. The lowest dose used in this experiment is one of the lowest that has been shown to have an effect on any fish behaviour (see table 2), showing that fish behaviour can be affected at concentrations that may occur in polluted surface waters. Thus, feeding behaviour in the three-spined stickleback appears to be a very sensitive endpoint for SSRI effects in fish. While Paper I appears to be the first study which investigates the effects of citalopram on fish feeding behaviour, the SSRI fluoxetine has previously been shown to have similar effects (Mennigen et al., 2009; Weinberger and Klaper, 2014). In this study, the number of attacks on food was used as a measure of food intake. This measure is somewhat problematic because there is no guarantee that it is correlated to actual food intake. It may also be correlated to aggression, which could influence the results since SSRIs are known to reduce aggression. Indeed, despite a significantly decreased number of attacks, there was no significant difference in weight between exposed fish and controls by the end of the experiment. Nonetheless, since the results in this study are consistent with previously reported results of exposure to other SSRIs in fish (Mennigen et al., 2009), as well as results of studies on the effects of citalopram on rodents, the results are likely to be valid. While the exact mechanism behind the effect of serotonin on feeding behaviour has not been clarified, in mammals satiety appears to be mediated by the 5-HT\textsubscript{1B} and the 5-HT\textsubscript{2C} receptors in a complimentary fashion (Voigt and Fink, 2015).
**4.2 Anxiolytic effects (Paper II)**

Anxiolytic effects are perhaps what comes to the mind of most scientists when SSRIs are mentioned, which is natural since anxiolysis is what SSRIs were primarily designed for. In Paper II, female three-spined sticklebacks were exposed to 0 μg/l, 1.5 μg/l or 15 μg/l citalopram for three weeks after which they were subjected to a novel tank test and a novel object test. Fish exposed to 1.5 μg/l made significantly more transitions to the upper half of the aquarium and spent more time there than control fish. Fish exposed to 15 μg/l exhibited significantly less freezing than controls. Locomotor activity was significantly enhanced in both treated groups. In the novel object test, exposed fish spent significantly more time in the proximity of the novel object than control fish. The results in Paper II show clearly that citalopram has anxiolytic effects on the three-spined stickleback. The concentrations used are however, approximately one order of magnitude higher than what is commonly found in polluted surface waters. Nevertheless, at the time of publishing this study was the first to show that waterborne citalopram could have anxiolytic effects in wild-caught fish at concentrations as low as 1.5 μg/l. This is largely in accord with results reported in other studies of SSRI effects on fish behaviour, which frequently report anxiolytic effects of SSRI exposure. Some examples of such effects are diminished response to conspecific alarm pheromones (Barbosa et al., 2012; Barry, 2012), reduced white avoidance in the scototaxis test (Maximino et al., 2011), increased exploratory behaviour in the novel tank test (Egan et al., 2009; Sackerman et al., 2010; Olsén et al., 2014), and reduced distance swum when confronted with a mock predator (Weinberger and Klaper, 2014). Locomotor activity in fish is commonly suppressed after both acute (Winder et al., 2012) and chronic (Clements and Schreck, 2007) SSRI exposure, which is contradictory to our results. In crabs, however, increased locomotor behaviour as a result of fluoxetine exposure has been demonstrated (Mesquita et al., 2011). Locomotor activity is a complicated measure because when it is measured in a novel environment, as was the case in this study and most others, it is related to exploratory behaviour, and it is often impossible to know if changes in locomotor behaviour are due to a specific effect on locomotor behaviour or a side-effect of increased exploratory behaviour. This is especially true when locomotor behaviour is increased as it was in Paper II. At least one study suggests that dopamine levels are a possible mediator of locomotor response to fluoxetine exposure (Shishkina et al., 2006). However, the dopaminergic effects of citalopram are generally regarded as much less pronounced than those of fluoxetine (Pozzi et al., 2001).

**4.3 Effects of developmental exposure (Paper III)**

In addition to its role as a signalling substance in the nervous system, serotonin is also involved in embryonic development. Developmental SSRI exposure has been
shown to cause a range of behavioural abnormalities in mammals (Ansorge, 2004; Maciag et al., 2005; Iniguez et al., 2010; Rodriguez-Porcel et al., 2011; Simpson et al., 2011; Iniguez et al., 2014; Svirsky et al., 2016; Kiryanova et al., 2016) but studies on similar effects in fish appear to be completely absent. To address this knowledge gap, we studied the effects of citalopram exposure during development in three-spine sticklebacks. Seven sibling groups were raised from seven different couples. Each sibling group was split in two and one half of each sibling group was exposed to 1.5 μg/l citalopram in the ambient water for 30 days starting at two days post fertilisation (dpf). Two dpf is the development stage at which serotonergic neurons start to appear in the developing stickleback embryo (Ekström, 1994). The other half of the sibling group received a corresponding amount of MilliQ water, yielding a paired experimental design. After approximately 100 days of remediation in clean water, the fish were tested for stress related changes in behaviour as well as behavioural changes related to feeding and aggression. Fish of both sexes were used but the fish were not sexually mature when behavioural testing took place. The results were mostly opposite to what is commonly seen in adult fish during on-going citalopram exposure. The number of attacks on food was significantly higher in the exposed fish, and they were more aggressive than control fish when confronted with a mirror image. Locomotor activity was significantly suppressed in exposed fish, and in the scototaxis test the number of transitions to the white side of the aquarium in the scototaxis test was lower in exposed fish. The novel tank test yielded no statistically significant effects of treatment.

The effects of acute and chronic SSRI exposure on fish behaviour are by now reasonably well understood although the ecological consequences remain to be investigated. The results in Paper III represent the next step in the investigation of detrimental effects of SSRI as pollutants and as such, they should be interpreted with some care. Since Paper III is the first paper on the subject, it can not be directly compared to other studies and has to be compared to studies on rodents. The results in Paper III are partly, but not entirely, consistent with the effects of developmental exposure to SSRIs that have been observed in rodents. The reduced exploratory behaviour observed in the scototaxis test in Paper III is consistent with the observations of reduced exploratory behaviour made by Ansorge (2004), and the increased aggression seen in the sticklebacks after exposure is consistent with the observations of increased aggression in exposed male mice made by Kiryanova (2016) and Svirsky (2016). The results are not consistent, however, with the observations of increased locomotor behaviour in exposed rats made by Maciag (2005) or the reduced food intake observed by Ansorge (2004). These differences may reflect either differences in experimental methodology or differences between fish and rodents in the way that serotonin regulates behaviour. For example, it can be hypothesised that feeding may be more closely related to aggression in the three-spined stickleback than in rodents. This may be especially true if sticklebacks, which are known to react aggressively to the colour red, are fed with food of that colour as was
the case in Paper III. Ansorge (2004) found that the behavioural effects of developmental exposure were closely mimicked by serotonin re-uptake transporter knockout mice and that developmental exposure to fluoxetine had no effect on behaviour in such mice and Maciag et al. (2005) found decreased expression of the re-uptake transporter and a reduction in the rate-limiting serotonin synthetic enzyme tryptophan hydroxylase in the dorsal raphe. It seems reasonable to conclude that the predominant mechanism behind the behavioural effects of developmental exposure to SSRIs is reduced expression of the serotonin re-uptake transporter (5-HTT). However, the effects of developmental exposure are alleviated by re-exposure to SSRIs (Iñiguez et al., 2010). If 5-HTT underexpression was the mechanism, the serotonergic tone should already be high and thus no alleviation should take place. Besides affecting 5-HTT expression, developmental exposure to fluoxetine also affects brain sexual differentiation (Rayen et al., 2013), and developmental exposure to citalopram and fluoxetine changes cortical network organisation and neuronal function (Simpson et al., 2011; Smit-Rigter et al., 2012). It may therefore be difficult to distinguish cause from effect, and changes in cortical organisation may be an effect of reduced 5-HTT expression and/or produce the behavioural effects that have been observed.

### 4.4 Combined effects of citalopram and 17α-ethinyl estradiol (Paper IV)

In the environment, SSRIs typically occur in complex mixtures with different SSRIs, other pharmaceuticals and personal care products. Some of those substances have the potential to modulate fish behaviour. This modulation can be anxiolytic as in the case of SSRIs (Kellner et al., 2016) and benzodiazepines (Brodin et al., 2013). Others can be anxiogenic, such as EE₂, the main active ingredient in many contraceptives (Hallgren et al., 2011; Reyhanian et al., 2011). In Paper IV, zebrafish of both sexes were exposed to either the anxiogenic endocrine disruptor EE₂ (0.1 and 0.5 ng/l), citalopram (0.1 and 0.5 μg/l), a low exposure combination (0.1 ng/l EE₂ + 0.1 μg/l citalopram), or a high exposure combination (0.5 ng/l EE₂ + 0.5 μg/l citalopram). 0.1 ng/l equals the predicted NOEC for EE₂. There were few effects of any of the treatments in the females, and the result in the male group were ambiguous. In the scototaxis test, EE₂ alone increased anxiety levels in males. While citalopram alone did not have any effects on anxiety levels as measured in the scototaxis test in Paper IV, it did abolish the anxiogenic effects of EE₂ in the combined treatments. In the novel tank test, 0.1 ng/l EE₂ unexpectedly and significantly decreased the latency to the first transition to the upper half. 0.1 μg/l citalopram increased the number of transitions to the upper half. The low exposure combination group (0.1 ng/l EE₂+0.1 μg/l citalopram) exhibited a longer latency to the upper half of the aquarium than both the citalopram and EE₂ groups. The low exposure combination group also exhibited fewer transitions to the upper half than both single-substance exposures
as well as the control group, showing a combinatory effect of the two substances. In
the shoaling test, the male fish exposed to 0.1 μg/l citalopram performed fewer tran-
sitions away from the peer group compared to both control and the low exposure
combination group. The high exposure females exposed to only EE2 performed
fewer transitions away from the peer group. The data in Paper IV show that the
stress axis in zebrafish is extremely sensitive to the anxiogenic effects of EE2,
especially in males. They also imply that the predicted no adverse effect level of EE2,
0.1 ng/l, (Caldwell et al., 2012) may have to be adjusted downwards. Paper IV
indicates that there are combinatory effects of EE2 and citalopram. However, in the
scototaxis test citalopram appears to ameliorate the anxiogenic effects of EE2 while
in the novel tank test citalopram appears to amplify those effects. Those are very
puzzling results and indicate that the effects of EE2 may be context dependent.
Novel tank and scototaxis have previously been shown to be complementary to each
other rather than measuring the same behavioural syndrome. While novel tank
behaviour depends on a stress factor (a novel environment) and is subject to habitu-
ation, aversion to bright colours is evident regardless of stress, or is inherently
stressful, in zebrafish and no habituation occurs (Blaser et al., 2010).

4.5 Environmental risk analysis

Previous NOEC or LOEC values for exposure to citalopram in aquatic organisms
are scarce in literature. Henry et. al. (2004) estimated the LC50-value in C. dubia
over 48 hours to be 3.9 mg/l and the LOEC for an effect on reproduction to be 4
mg/l in a chronic (8 days) exposure paradigm. Thus, the values published by Henry
et. al. (2004) are more than 20,000 times higher than the concentrations that were
found to have an effect on fish behaviour in Paper I in this thesis. This difference
may depend partly on the choice of endpoint and exposure time. It may also be a
matter of choice of model organism since the behaviour of C. dubia might not be
complex enough to reliably reflect the responses of higher organisms. At the other
end of the spectrum, an endpoint which has been shown to be extremely sensitive is
foot detachment from the substrate in the freshwater snail Leptoxis carinata, which
occurs at levels as low as 405 pg/l (Fong and Hoy, 2012).

In Paper I, feeding behaviour was found to be affected at 150 ng/l and in Paper
IV anxiolysis was induced at 100 ng/l. While it should be noted that those are only
LOEC values and contain no information as to how low the levels need to be to
exclude an effect, the predicted no effects concentrations (PNECs) estimated by
Ahlford (2007) come relatively close to our values, and a PNEC from literature with
a safety factor of 1000 results in 4.9 μg/l while a PNEC derived from a QSAR model
and with a safety factor of 50 amounts to 0.004 μg/l.
5. Summary and conclusion

Natural selection favours the individuals that are best at spreading their genes (Darwin, 1859), thus ensuring long term population survival. We know the ability to reproduce one's genes as fitness. This is essentially achieved by maximising energy intake and long term offspring production while minimising the risk to fall prey to other organisms. However, those are often conflicting goals since active foraging will increase food intake but is likely to expose an organism to the risk of predation. Thus, to maximise fitness any individual organism living in an environment where there are predators has to strike a balance between the risk to become prey and the need to find prey and boldness is an important ecological parameter for individual and population fitness. Not surprisingly, boldness has been shown to affect the outcome of competition for food at an individual level (Webster et al., 2008). Like most other organisms, a fish has to continuously weigh risks against potential benefits and consequently, starved fish take higher risks to obtain food than well-fed fish (Godin and Crossman, 1994; Herczeg and Välimäki, 2011). The same kind of reasoning about trade-offs can be applied to aggression. This may be especially true for shoaling fish, where too much aggression may increase the chance to gain a high position in the shoal hierarchy which brings advantages such as increased access to food (Sneddon et al., 2006) and mates but which may also increase the risk to get hurt or killed in fights against conspecifics. In short, fish and other organisms need to constantly balance the various selective pressures to maximise fitness.

Because citalopram and other SSRIs can modulate behaviour, the main concern about SSRIs as pollutants is that exposed fish may either be less anxious and thus take more chances which may lead to predation, or that the selective pressures that select for strong, genetically fit individuals may be weakened. A possible example of sub-ideal SSRI-induced behaviour is a decreased propensity to stay close to the bottom or otherwise seek shelter which has been shown to happen at environmental concentrations in Paper IV, and at slightly higher concentrations in Paper II, in this thesis. Ignoring risks may increase food intake but at the price of higher predation risk. While it has not been shown in this thesis, aggression is generally thought to be weakened by SSRI exposure, which could reduce competition for food and mates and therefore weaken the selection pressure for strong individuals. In addition to the effects on boldness and aggression, SSRI-exposed fish make fewer attacks on food than non-exposed fish. As with the effects on boldness, this effect manifests itself at concentrations that may be found in polluted surface waters. In this thesis, it was not possible to correlate the lower number of attacks on food to actual reduced
weight, but this has been done successfully by others (Mennigen et al., 2009) which lends credibility to the observation. Feeding is of course vital for fitness and is interconnected with boldness. Bold sticklebacks have been shown to outcompete their shy peers for food and to grow faster (Ward et al., 2004). In conclusion, the main worries so far about SSRI effects on fish have been weakened natural selection and altered ecological structures due to more risky behaviour in prey species such as the three-spine stickleback. However, while all those behavioural effects can be shown in the lab, most of it remains to be validated in the field. This is likely because such field trials would be very costly and technically difficult.

So far the effects of SSRIs. However, under real world conditions citalopram and other SSRIs usually occur in complex mixtures of SSRIs and other pharmaceuticals, personal care products and industrial chemicals which may antagonize or enhance the anxiolytic properties of citalopram. Their effect may also be enhanced or diminished by citalopram as shown in Paper IV, where citalopram had little effect on zebrafish behaviour by itself but counteracted the anxiogenic properties of EE2 as shown by the scototaxis test, while enhancing the anxiogenic effects as shown by the novel tank test. Together, those experiments demonstrate that the effects of such mixtures may be context dependent and point out the difficulties in predicting the results of pharmaceutical cocktails. While it is not feasible to test every possible cocktail scenario, careful investigation of the interaction between different systems (for example the reproductive neuroendocrine axis and the stress axis) may provide modelling tools that could allow prediction of effects from not yet tested mixtures.

All research concerning effects of SSRIs on fish so far has focused on effects in adult animals. However, the serotonergic system which is the target for SSRIs, is deeply involved in the embryonic development of vertebrates and appears to play an important role for the developing nervous system (Simpson et al., 2011; Smit-Rigter et al., 2012). Socio-behavioural abnormalities as a result of perinatal SSRI exposure have been demonstrated in both rodents (Ansorge, 2004; Maciag et al., 2005; Iniguez et al., 2014) and humans (Oberlander et al., 2010; Klinger et al., 2011).

Paper III is the first study to show the effects of developmental SSRI exposure in fish. The ecological implications of such effects are not clear however, and many questions remain to be answered. For example, the experiment in Paper III in this thesis simulates the scenario of anadromous fish which spend their juvenile stages in rivers where they may be exposed to high levels of SSRIs but spend most of their adult lives in the open sea. Fish species with this kind of life history may be especially vulnerable to the detrimental effects of developmental SSRI exposure but what about stationary fish that stay in rivers their entire life and which may therefore be exposed throughout their life? Is it possible that the behavioural syndrome seen in developmentally exposed fish and rodents represents an adaptation to the presence of an SSRI, i.e. would the fish have behaved normally if the exposure had continued? The research on effects of developmental exposure is still in its infancy, both from a clinical and environmental point of view. Although human health is
not the focus for this thesis, the results from Paper III are worrying considering the similarities between the piscine and human serotonergic systems.

To judge the environmental risk of a substance, LOEC and NOEC values are often compared to measured environmental concentrations (MECs). However, LOEC and NOEC values for waterborne citalopram are scarce in literature. Some of the ecotoxicological studies that do exist are focused on classical toxicological endpoints like LC50 and reproduction in plankton (table 2) which is clearly unsuitable since SSRIs exert their effect on the vertebrate serotonergic system at concentrations far below the lethal dose, and plankton don’t possess any advanced nervous system on which the SSRIs can act. In this study, it has been shown that effects on stress related behaviours and feeding behaviour in the three-spined stickleback arise at a fraction of previously reported NOECs in *C. dubia* and most other organisms. Effects from developmental exposure (see appendix) also occurs at concentrations that are lower than most previously reported NOECs (see table 2). The results in this thesis compared to previous results reported in literature highlight the importance of choosing the correct (i.e. the most sensitive) organism and endpoint.

While the reported LOEC of 100 ng/l for anxiolytic effects which is reported in Paper IV and the LOEC 150 ng/l for feeding behaviour from Paper I are lower than the majority of previously reported LOECs and less than a tenth of any previously reported LOEC for fish, a comparison with measured environmental values paints an ambiguous picture. Table 1 lists 18 different measured concentrations of citalopram in STP effluents, compares them to the LOEC found in this study and calculates the risk quotient, RQ. No compensation for dilution or any safety factor has been introduced. The risk quotient is defined as the measured environmental concentration (MEC) divided by the LOEC. An RQ above 1 indicates a risk of effects. In table 1, 13 out of the 18 studies contain values that give rise to RQs above 1. While this is a crude measure which does not take into account dilution or the additive effects of other SSRIs, the results in table 1 are clearly enough to cast doubt on the environmental safety of citalopram and other SSRIs. It must be concluded that at least in environments where the dilution of STP effluents or other forms of SSRI pollution is limited, behavioural effects on fish are very likely. It should also be kept in mind that no NOEC was found in any of the studies included in this thesis and the lower limit for SSRI effects on fish is therefore still not known.
6. Acknowledgements

This thesis would not have been possible without my many outstanding collaborators. First of all, many thanks go to my supervisors Håkan Olsén, Inger Porsch-Hällström and Stefan Hallgren. Secondly, without my closest PhD colleague Tove Porseryd, this thesis would not have been what it is. Thank you for all the rewarding hours in the dissection room. A special thanks also goes to Patrik Dinnetz, whose insights into the world of statistics and the R software have been invaluable. I would like to thank Kristina Volkova, Nasim Caspillo, Josefine Larsson and all my other colleagues who have made my time as a PhD student at Södertörn University so much more fun than it would otherwise have been. Finally, I would like to thank my life companion Catarina Ekenäs who has made this thesis possible by supporting me and who has also drawn the cover page for this thesis.
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8. Appendix

Appendix 1: Effects of treatment in paper I-III. ↑ signifies a statistically significant increase, ↓ signifies a decrease and – signifies no statistically significant difference from control.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Behavioural test</th>
<th>Treatment</th>
<th>Effect</th>
<th>Paper</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of attacks on food</td>
<td>Feeding test</td>
<td>CIT 1.5 µg/l</td>
<td>↓</td>
<td>I</td>
<td>Effect within a week</td>
</tr>
<tr>
<td>Number of attacks on food</td>
<td>Feeding test</td>
<td>CIT 0.15 µg/l</td>
<td>↓</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Number of attacks on food</td>
<td>Feeding test</td>
<td>CIT 1.5 µg/l</td>
<td>↓</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Number of transitions to upper half</td>
<td>Novel tank</td>
<td>CIT 1.5 µg/l</td>
<td>↑</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Time spent in upper half</td>
<td>Novel tank</td>
<td>CIT 1.5 µg/l</td>
<td>↑</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Latency to upper half</td>
<td>Novel tank</td>
<td>CIT 1.5 µg/l</td>
<td>-</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Number of freeze bouts</td>
<td>Novel tank</td>
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<td>-</td>
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<td></td>
</tr>
<tr>
<td>Total time spent freezing</td>
<td>Novel tank</td>
<td>CIT 1.5 µg/l</td>
<td>-</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Number of transitions to upper half</td>
<td>Novel tank</td>
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<td>-</td>
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<td>Time spent in upper half</td>
<td>Novel tank</td>
<td>CIT 15 µg/l</td>
<td>-</td>
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<td></td>
</tr>
<tr>
<td>Latency to upper half</td>
<td>Novel tank</td>
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<td>-</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Number of freeze bouts</td>
<td>Novel tank</td>
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<td>↓</td>
<td>II</td>
<td></td>
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<tr>
<td>Total time spent freezing</td>
<td>Novel tank</td>
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<td>↓</td>
<td>II</td>
<td></td>
</tr>
<tr>
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<td>Novel tank</td>
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<td>↑</td>
<td>II</td>
<td></td>
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<tr>
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<td>Novel tank</td>
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<td>↑</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Number of entries into outer circle</td>
<td>Novel object</td>
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<td>↑</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Number of entries into middle circle</td>
<td>Novel object</td>
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<td></td>
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<td>Developmental exposure</td>
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<td>III</td>
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<td>III</td>
<td>Developmental exposure</td>
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Appendix 2: Effects of treatment in Paper IV. ↑ signifies an increase, ↓ signifies a decrease and – signifies no statistically significant difference from control. Female fish were part of the study in Paper IV but did not differ from control for any variable. Continued in appendix 3.

<table>
<thead>
<tr>
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<th>Behavioural test</th>
<th>Treatment</th>
<th>Effect</th>
<th>Paper</th>
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<tr>
<td>Number of trans. to upper half</td>
<td>Novel tank</td>
<td>EE2 0.1 ng/l</td>
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</tr>
<tr>
<td>Time spent in upper half</td>
<td>Novel tank</td>
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<td>Novel tank</td>
<td>EE2 0.1 ng/l</td>
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<td>Male fish</td>
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<tr>
<td>Number of trans. to upper half</td>
<td>Novel tank</td>
<td>CIT 0.1 µg/l + EE2 0.1 ng/l</td>
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<td>Time spent in upper half</td>
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Appendix 3: Results in Paper IV continued.

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<th>Treatment</th>
<th>Effect</th>
<th>Paper</th>
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Selective serotonin re-uptake inhibitors (SSRIs) are a group of antidepressants that are, due to their efficacy and mild side effects, a first-line treatment against a range of psychological illnesses. However, they are also emerging pollutants and commonly found in effluents from sewage treatment plants and in surface waters in many parts of the world. The prime neurological target of SSRI, the serotonergic system, is not unique to mankind but is virtually identical throughout the vertebrate clade, making many animals susceptible to SSRI effects. The serotonergic system is involved in mood, a wide range of behaviours and various processes during embryonic development and it is therefore suspected that SSRIs may alter the behavioural profile of exposed animals.

This thesis explores the effects of SSRIs on various aspects of fish behaviour, from effects on boldness and feeding behaviour via effects of pharmaceutical cocktails to effects of developmental exposure.

Martin Kellner was born in 1978 and has a master of science degree in biology from Uppsala university. He's fascinated by how we as human beings interact with our environment and how environmental damage can be minimised in this interaction. Pharmaceuticals were specifically designed to modulate biological systems, a property he thinks makes them especially exciting.