ASSESSMENT OF ENDOCRINE DISRUPTING ACTIVITIES IN THE AQUATIC ENVIRONMENT

Carina Björkblom

Academic dissertation

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Opponent Professor Helmut Segner
University of Bern, Centre for Fish and Wildlife Medicine
Department of Animal Pathology
Bern, Switzerland

Department of Biology, Åbo Akademi University
Finnish Graduate School in Environmental Science and Technology
Turku, Finland
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Carina Björkblom

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From the Department of Biology, Åbo Akademi University and the Finnish Graduate School in Environmental Science and Technology

Reviewed by

Professor Leif Norrgren
Department of Biomedical Sciences and Veterinary Public Health
Division of Pathology, Pharmacology and Toxicology
Swedish University of Agricultural Sciences
Uppsala, Sweden

Dr Stephen Feist
Group Head: Aquatic Animal Disease
Cefas Weymouth Laboratory
Weymouth, UK

Opponent

Professor Helmut Segner
University of Bern
Centre for Fish and Wildlife Medicine
Department of Animal Pathology
Bern, Switzerland

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The best scientist is open
to experience and begins
with romance – the idea that
anything is possible.

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This thesis is based on the following original publications and manuscripts, which are referred to in the text by the Roman numerals. The original publications have been reproduced with permission of the copyright holders.

I Björkblom, C., Mustamäki, N., Olsson, P.E., Katsiadaki, I., Wiklund, T. Assessment of reproductive biomarkers in three-spined stickleback (*Gasterosteus aculeatus*) from sewage effluent recipients. Manuscript.

II Björkblom, C., Högfors, E., Salste, L., Bergelin, E., Olsson, P.E., Katsiadaki, I., Wiklund, T. Estrogenic and androgenic effects of municipal wastewater on endpoint biomarkers in three-spined stickleback. Environmental Toxicology and Chemistry, in press.

III Björkblom, C., Olsson, P.E., Katsiadaki, I., Wiklund, T., 2007. Estrogen- and androgen-sensitive bioassays based on primary cell and tissue slice cultures from three-spined stickleback (*Gasterosteus aculeatus*). Comparative Biochemistry and Physiology, Part C, Toxicology and Pharmacology, 146, 431-442.

IV Björkblom, C., Salste, L., Katsiadaki, I., Wiklund, T., Kronberg L., 2008. Detection of estrogenic activity in municipal wastewater effluent using primary cell cultures from three-spined stickleback and chemical analysis. Chemosphere, 73, 1064-1070.

ABBREVIATIONS

11-KT	11-ketotestosterone
AR	Androgen receptor
Calcein-AM	Calcein-acetoxymethyl ester
CF	Condition factor
CYP	Cytochrome P450
Da	Dalton
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DHT	5α-dihydrotestosterone
E2	17β-Estradiol
EE2	17α-Ethynylestradiol
EDC	Endocrine disrupting compound
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Estrogen receptor
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GSI	Gonadosomatic index
GtH	Gonadotropin
HSI	Hepatosomatic index
LC-MS	Liquid chromatography-mass spectrometry
MT	Methyltestosterone
MTBE	Methyl tert-butyl ether
NSI	Nephrosomatic index
OECD	The Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PFA	Paraformaldehyde
SDS	Sodium dodecyl sulphate
SPE	Solid phase extraction
STP	Sewage treatment plant
T	Testosterone
TBT	Tributyltin
TIU	Trypsin inhibitor units
VTG	Vitellogenin

ABSTRACT

Endocrine disruption in the aquatic organisms has been reported from many regions all over the world and has often been linked to different types of effluents. The aim of this thesis was to investigate whether endocrine disruption related to the release of estrogenic or androgenic substances in wastewater effluent is significant in Finnish waters. The thesis describes laboratory based exposure experiments and a field survey conducted with adult three-spined stickleback (*Gasterosteus aculeatus*) as model species. This thesis also describes rapid *in vitro* screening methods for detection of endocrine disrupting activities, complementing animal models, and chemical analysis for identification of active compounds in wastewater effluent. Laboratory exposure experiments found the three-spined stickleback to be sensitive to waterborne estrogenic and androgenic steroid substances. Exposure to estrogens was demonstrated through pronounced expression of the yolk protein precursor vitellogenin while exposure to androgens was monitored through analysis of spiggin expression, a unique androgen biomarker present only in the stickleback. Wastewater effluent was shown to exert estrogenic effects in sticklebacks. The field survey along the Finnish Baltic Sea coastline revealed signs of estrogenic disruption in stickleback in receiving areas of sewage treatment plant effluents. No clear androgen activity was observed in wastewater effluent either in laboratory based exposures or in the field survey. The estrogen sensitive *in vitro* assay based on primary hepatocyte cell cultures was able to predict the responses of wastewater effluent in fish *in vivo*. The androgen sensitive *in vitro* assay based on kidney cell and tissue slice cultures also successfully detected androgenicity of pure androgens tested. However, municipal wastewater effluent showed no androgen activity when tested *in vitro*, corresponding to results *in vivo*. Steroidal estrogens were detected in wastewater effluent by chemical analysis. Interestingly, these estrogens were responsible for only a minor part of the observed effects *in vivo* and *in vitro* and the results imply that the examined wastewater effluent contain compounds with estrogenic activity, other than the steroidal estrogens measured.

SVENSK SAMMANFATTNING

Hormonstörande ämnen är syntetiska eller naturliga ämnen som stör organismers hormonsystem och bidrar till könsförvirring och sterilitet. Sådana ämnen kommer i ökande takt ut i vattenmiljön genom rester av läkemedel, bekämpningsmedel och industriprodukter. Eftersom det finns många likheter mellan hormonsystemen hos människan och övriga ryggradsdjur kan till exempel fiskar användas som modellsystem för att underöka detta problem. I tidigare undersökningar har man funnit hormonella störningar, bl.a. feminisering och maskulinisering hos fiskar som utsatts för avfallsvatten från kommunala reningsverk eller avfallsvatten från pappersindustrin.

Målet med denna avhandling var att undersöka om renat avfallsvatten från kommunala reningsverk längs Finlands kust innehåller hormonstörande ämnen i sådana mängder att de kan försorsaka hormonstörande effekter på fisk. Målet i denna avhandling var också att utveckla cellulära testsystem baserade på fiskceller, eftersom behovet av tillförlitliga och kostnadseffektiva cellbaserade tester för att underlätta riskbedömningen av hormonstörande ämnen är mycket stort för tillfället. Som testsystem har storspiggen använts, som besitter flera användbara biomarkörer för att mäta hormonstörande ämnens påverkan.

Resultaten tyder på att problemen med hormonstörande påverkan på fisk inte är lika utbredda i Finland som i många andra europeiska länder. Detta beror troligtvis på att finska reningsverk har effektiva reningstekniker som reducerar mängden hormonstörande ämnen, eller på att utspädningen av avloppen i recipienterna är större än i många andra länder. Dock kan problemen inte helt uteslutas eftersom vissa feminiserande (estrogena) effekter kunde observeras hos fisken i de undersökta recipienterna utanför kommunala reningsverk. I kontrollerade laboratorieförsök där storpsiggar exponerades för kommunalt avloppsvatten uppmättes även här effekter som tyder på förekomst av estrogener i avloppsvattnet. De cell-baserade testsystemen klarade av att förutspå hormonella effekter hos hel fisk och kan därför vara mycket användbara i fortsatta studier av hormonstörande ämnens verkningsmekanismer i preliminära toxicitetsbedömningar.

1. INTRODUCTION

Protection of the environment from contamination by natural or synthetic substances emitted from human activities has become a matter of growing concern during the previous two decades. A wide range of contaminants including pesticides, detergent residues, plasticisers, natural and synthetic estrogens, polybrominated diphenyl ethers, polychlorinated biphenyls, disinfection by-products and others are present in wastewater and the waters receiving it. Some of these contaminants have the potential to disrupt the normal functions of endocrine systems in higher organisms thus cause health effects in wildlife and humans. These compounds are commonly referred to as endocrine disrupting compounds (EDCs). Studies in Europe, North America, Japan and Australia have reported the presence of EDCs in sewage treatment plant effluents, which could affect physiological and reproductive function in exposed fish consistent with exposure to hormonally active chemicals. The observed abnormalities vary from subtle changes to permanent alterations including disturbed sex differentiation and with feminised or masculinised sex organs, changed sexual behaviour and altered immune function. The occurrence of EDCs in rivers and receiving environments situated near sewage treatment plants raises concerns over the removal efficacy of these compounds by conventional treatment processes.

Knowledge about causes and effects of endocrine disruption is comprehensive for fish. This is because their early identification as a group showing endocrine disruption in the field, their marked exposure to contaminants via both oral and branchial routes, their relatively well understood endocrinology and their amenability to experimental manipulations. Fish possess endocrine systems which mirror in many respects more evolved vertebrates and therefore have the potential to act as sentinels for possible effects on other vertebrates including humans. Although a causal relationship between exposure to environmental pollutants and adverse effects on human reproductive health has not been established, endocrine disruption remains a topic to which much research effort is dedicated.

Biomarkers in fish have been used extensively in monitoring as well as in risk assessment. Biomarkers are often used as early warning signals for detection of effects at the population level. The use of biomarkers in fish for simultaneous assessment of xenoestrogenic and xenoandrogenic effects can dramatically result reduction in the number of test animals, an important factor from an ethical viewpoint. Development of rapid *in vitro* screening methods for screening, particularly EDCs, would also be of great importance.

2. REVIEW OF THE LITERATURE

The issue of endocrine disruption in both humans and wildlife was raised in the 1990s and quickly attracted attention of researchers, regulators, and the public. The aquatic environment is particularly susceptible to pollution for both anthropogenic and natural chemicals that are released to the watercourse via waste effluents and agricultural activities. Although reproductive abnormalities in fish were reported as early as the 19th century, the link between these observations and the presence of chemicals with endocrine modulating activity were not made until two decades ago. Today, several field and laboratory studies have shown induction of adverse effects in wildlife upon exposure to endocrine disrupting compounds (EDCs). The importance of the issue is highlighted by the large number of studies linking human adverse effects and exposure to EDCs.

2.1. Endocrine disruption

Some chemicals, called endocrine disruptors, can interfere with the hormonal system. Endocrine disruption is not considered a toxicological end point per se, but a functional change that may lead to adverse effects. The endocrine disruption hypothesis claims that synthetic chemicals as well as naturally occurring substances in the environment disrupt the normal functions of the endocrine system and these hormones in humans and wildlife (Colborn and Clement, 1992; McLachlan, 2001; Matthiessen, 2003). An endocrine disruptor is defined as *"an exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that is responsible for the maintenance of homeostasis, reproduction, development and/or behaviour"* (Kavlock, 1996) or *"an exogenous substance that causes adverse health effects in an intact organism or its progeny, subsequent to changes in endocrine function"* (European Commission, 1996). Endocrine disruption occurs when there is negative interference or permanent adverse consequences beyond the range of everyday fluctuations of hormone levels.

2.1.1. Endocrine disrupting chemicals

Endocrine disrupting compounds encompass a variety of chemical classes including natural and synthetic hormones, plant constituents, pesticides, compounds used in the plastics industry and in consumer products, other industrial by-products and pollutants. EDCs are often pervasive and widely dispersed in the environment. Some EDCs are persistent and lipophilic and are sequestered in adipose tissue and secreted in milk. These can be transported long distances across national boundaries and have been found in virtually all regions of the world. Other EDCs are rapidly degraded in the environment or in the human body. However, even short exposure times to EDCs might be fatal at

critical periods of development. The group of hormones believed to be most susceptible to the action of endocrine active chemicals are sex steroids. Contaminants that have received the most attention from the research world are those that are capable of eliciting responses typically induced by sex steroid hormones. The aquatic environment is a sink for chemical substances and therefore, aquatic animals are particularly endangered by the action of EDCs (Tyler et al., 1998). To date, most research on endocrine disruption in fish has been focused on the action of estrogen-active compounds, i.e. compounds that exert their activity through ligand binding to the estrogen receptor (ER). However, other hormonal receptors such as the androgen receptor (AR), thyroid receptor, and aryl hydrocarbon receptor (AhR) can be impacted as well (Bolander, 1994).

2.1.2. The action of EDCs

Most focus has been directed on the effects of EDCs whose action may be partially mediated by binding to steroid hormone receptors, but there are also many other potential mechanisms of action. EDCs can, at the cellular level, induce endocrine disruption via a number of routes that involve steroid receptor binding (agonists), blocking steroid receptor binding (antagonists), or by disrupting the biosynthesis and or metabolism of steroids (Sharpe and Irvine, 2004). Steroid hormone receptors are transcription factors regulating the expression of target genes (Truss and Beato, 1993). Steroid hormone receptors have traditionally been considered to act via the regulation of transcriptional processes, involving nuclear translocation and binding to specific response elements, and ultimately leading to regulation of gene expression. There are several experimental systems available for evaluating the interaction of exogenous or synthetic chemicals with hormone systems, particularly those that interact with the ER, AR, thyroid or AhR (Bolander 1994). However, interaction of chemicals with other receptor systems may also be important. These include the retinoid acid receptor, cytokine systems, and a number of so-called orphan receptors such as the peroxisome proliferators receptor system (IPCS, 2002).

The classical view that steroids act only through binding to a high affinity steroid receptor (Truss and Beato 1993) has been challenged. There is increasing literature regarding rapid, non-genomic actions of steroid hormones. These rapid effects of steroid hormones do not rely on gene transcription or protein synthesis. There is evidence to support steroid-induced modulation of cytoplasmic and cell membrane bound regulatory proteins, intra-cellular signalling cascades involving mitogen activated protein kinases, phosphotidylinositol 3-OH kinase or tyrosine kinases (Fischer, 2004). Steroid modulation of cell membrane bound ion channels and G-coupled receptors have also been reported (Simoncini and Genazzani, 2003). Another example of non-receptor mediated endocrine disruption is through inhibition of enzymes involved in steroid hormone metabolism. The levels of circulating steroids in biological systems are controlled by homeostatic feedback

loops. There are a battery of enzymes that influence their metabolism and elimination such as cytochrome P450-mediated hydroxylation, glucuronidation, methylation or sulfonation (Zhu and Conney, 1998).

Persistent, lipophilic organic pollutants often bioaccumulate in species at the top levels of the food chain. The pharmacokinetics of chemicals plays a major role in determining exposure (van Birgelen and van den Berg, 2000). Therefore, it is important to remember that EDCs can exert their hormonal activities either because of their intrinsic activity or through their metabolic products. There is also data suggesting that EDCs may not induce adverse effects when administered singly at a low dose, but that low dose effects occurs after exposure to a multi-component mixture of chemicals (Silva et al., 2002).

2.1.3. The effect of EDCs on wildlife

Wildlife studies provide early warnings about possible harmful effects to humans from exposure to EDCs. It is important to note that studies on the potential effects of EDCs on wildlife have mainly focused on individuals rather than whole populations or communities of animals. Generally, wildlife can be feminised, masculinised, or reproductively suppressed by EDCs. Evidence that EDCs affect wildlife comes from both invertebrates and vertebrates including mammals, birds, alligators and fish.

Invertebrates: The most complete example of endocrine disruption by an environmental contaminant is documented in molluscs exposed to tributyltin (TBT), an organotin compound found in antifouling paints. In the 1980s, the condition of imposex, the imposition of male sex organs onto females, was observed with increasing frequency in marine gastropods exposed to TBT (Bryan et al., 1986, Smith and McVeagh, 1991). Approximately 150 different species of gastropods have been affected by organotins worldwide (Matthiessen et al., 1999).

Mammals: Marine mammals, including seals, sea lions, porpoises, dolphins, and some whales have been found with high concentrations of organochlorine pollutants such as polychlorinated biphenyls (PCBs) and pesticides stored in their blubber (Le Boeuf et al., 2002; Tanabe 2002; Fossi et al., 2003). Marine mammals are top predators that bioaccumulate pollutants from their contaminated prey. Chemical loads of PCBs and polybrominated diphenyl ethers (a type of flame-retardant) are associated with altered reproductive and thyroid hormone levels. For example, in the 1980s, populations of Baltic seals declined markedly (Helle, 1983; Bergman and Olsson, 1985; ICES, 1992). Although overhunting and habitat destruction may have been contributing factors for these declining populations, it is generally accepted now that persistent pollutants (PCBs and DDE, a degradation product of DDT) adversely affected the reproductive performances of the females, resulting in the decline in seal numbers. Polar bears are ultra-top predators and as such, they can have very high concentrations of pollutants in

their blood and adipose tissue. In a heavily contaminated population of polar bears in Svalbard, Norway, a suite of hormonal changes has been found to correlate with the total blood level concentration of PCBs and organochlorine pesticides. Female bears with higher contaminant burdens had higher blood levels of progesterone (Haave et al. 2003). More heavily contaminated male bears had lower blood testosterone levels (Oskam et al. 2003). The implications of these hormonal disruptions are not fully understood. Whether low fertility is due to contaminant-related hormone problems or to differing population age structure and nutritional status is a source of continuing research and debate (Haave et al. 2003).

Birds: Eggshell thinning is primarily caused by DDE and can result in cracked or broken bird eggs. Other adverse reproductive effects caused by DDE, such as altered sex organ development, has also been observed in birds (Struger and Weseloh, 1985; Struger et al., 1985; Elliott et al., 1988). A well-accepted explanation for the eggshell thinning is that DDE blocks the cellular signal that allows the eggshell gland to deposit calcium in the shell (Bowerman et al., 2000; Dawson, 2000). The fate of the birds has improved after the ban of DDT in the United States and Europe. DDE concentrations have declined, eggshell thickness has improved in most species, and populations are recovering. However, DDT is still produced and used extensively in tropical regions to control malaria-carrying mosquitoes. Since these chemicals are long-lived and ubiquitous, bird life around the world is still at risk from the pollutants. Subsequent studies suggest that eggshell thinning continues to be a problem due to the high DDT content in eggs (Johnstone et al., 1996).

Reptiles: Alligators in Lake Apopka, Florida, USA provide one of the most publicized examples of EDC effects on a wildlife population. In 1980, high concentrations of dicofol, including its metabolites DDT, DDE and chloro-DDT, and other organochlorine compounds contaminated the lake after a chemical spill. Shortly thereafter, the population of alligators declined by 90% and the alligators had a variety of sex organ and other developmental abnormalities attributed to exposure to high levels of contaminants (Guilette et al., 1994).

Fish: Chemicals found in the waste outflows from pulp and paper mills and sewage treatment plants can affect reproduction and development in fish. Widespread feminisation of male fish has been found near freshwater municipal sewage outlets in England (Jobling and Sumpter, 1993; Jobling et al., 1998; 2002; Matthiessen et al., 2002; Kirby et al., 2004). Feminisation of marine fish species in offshore waters has also been observed (Allen et al., 1999; Bateman et al., 2004; Stentiford and Feist, 2005; Scott et al., 2007). Altered gonadal development, occurrence of ovotestis, induction of vitellogenesis in juvenile and male fish, reproductive abnormalities and reduced reproductive success have all been reported, and will be discussed later in this thesis. Alkylphenols and natural and synthetic estrogens are suspected to be causative factors for the feminising effects.

Masculinisation of female fish living downstream from paper mill wastewater outfalls has also been observed (Howell et al., 1980; Bortone et al., 1989). Pulp and paper mill effluents can suppress or inhibit reproductive capacity in male and female fish. The endocrine disrupting capability in the effluent remains even after eliminating chlorine bleaching compounds and improving treatment, which suggests that compounds present in the wood itself are responsible for the effects (Munkittrick et al. 1998).

2.1.4. The effects of EDCs on human health

A variety of human health concerns have been raised in relation to endocrine disruptors. There is evidence for a link between rising EDC levels and decreasing sperm counts, increases in testicular germ cell cancer, and increasing rates of cryptorchidism and hypospadias (reviewed in Toppari et al., 1996). A number of environmental chemicals have also been shown to affect the nervous system, ranging from motor impairment and memory loss to subtle behavioural changes. Increases in the incidence of certain cancers in many parts of the industrialised world are often cited as evidence that widespread exposure to the general population has had adverse impacts on human health. Cancers are especially prevalent at hormonally sensitive sites, such as the breasts, uterus, prostate, and testes, and coincide roughly with the increasing use and release of industrial chemicals into the environment (IPCS, 2002). There are questions being asked about whether EDCs with estrogenic properties or those that can prolong estrogenic activity could be causal factors in breast cancer occurrence (Davis et al., 1993). Over 500 weakly estrogenic EDCs have been identified (Brody and Rudel, 2003), but there is inadequate data to determine whether human exposure to EDCs poses any risk to reproduction (Sharpe and Irvine, 2004). The clearest example of an endocrine disrupter in humans is diethylstilbestrol, a synthetic estrogen prescribed in the 1950s and 1960s to pregnant women for the prevention of miscarriage. It was later found that some of the children who had been exposed in the uterus to diethylstilbestrol had developmental abnormalities. The relationship between early-life EDC exposure in humans and adult functioning is poorly understood. This is a concern because laboratory animal studies have indicated that early life stages may be especially sensitive to the effects of EDCs. Lack of controlled human exposure data is the major limiting factor in drawing conclusions about human reproductive health effects and links to EDCs.

2.1.5. Potential sources of exposure

Some EDCs may be released into the environment intentionally, as is the case with pesticides. Unintentional release of chemicals occurs throughout the life of chemical manufacturing, consumption and disposal. Dioxin-like contaminants are formed as by-products in a variety of industrial and combustion processes. Leakage from landfill areas and distribution via sewage sludge are also sources of EDC exposure (reviewed

in Campbell et al., 2006). Exposure to EDCs can occur via air, water, soil, sediment, food and consumer products. EDCs enter the organism by ingestion, respiration, or skin contact and are absorbed into the bloodstream (Crosby, 1998). If there is no active transport across the cell membranes, absorption is dependent on the ability of the chemical to cross cell membranes. Chemicals with molecular masses up to 1000 Da have been shown to be bio-available and to be transferred over biological membranes (El Dareer et al., 1987). Most environmental potential EDCs have masses in the range of 200-600 Da. The uptake of chemicals through the blood-brain barrier is of great importance and is influenced by the structure and polarity of the chemical.

2.1.6. Estrogens and anti-estrogens

Estrogens are a group of chemicals of similar structure mainly responsible for female sexual development and reproduction. They are produced mainly by the ovaries but also by the adrenal glands and adipose tissue. The principal estrogen is 17β-estradiol (E2). The ability of pesticides to act as estrogen agonists was shown 40 years ago (Bitman et al., 1968). Estrogenicity of anthropogenic chemicals, for example bisphenol A and DES, were first described in 1938 by Dodds and Lawson. Estrogen disrupters include pesticides, e.g. DDT and other chlorinated compounds, chemicals in some consumer and medical products, e.g. some plastic additives, and a number of industrial chemicals such as PCBs and dioxins. Many estrogen disrupters have been identified using *in vitro* assays. Several estrogen disruptors also display estrogen action *in vivo* including octylphenol, nonylphenol, bispenhol A, phystoestrogen, ethynylestradiol (EE2) and fungal mycotoxins. However, the affinity of the non-steroidal estrogen mimics for the estrogen receptor is usually several orders of magnitude lower than that of the natural ligand E2.

2.1.7. Androgens and anti-androgens

Androgens are chemicals responsible for the development and maintenance of the male sexual characteristics. They are structurally similar to estrogens, since estrogens are produced in the body from androgenic precursors. The principal androgen is testosterone (T), mainly produced by the testes. Endocrine disruption may occur through interference of environmental substances with androgen-signalling pathways and ligand binding to the androgen receptor (AR). A number of compounds are able to bind to the AR, including pharmaceuticals (flutamide), or pesticides such as vinclozolin. Several other substances have been shown to display AR-antagonist activity including the DDT metabolite DDE, fenitrothion, procymidone, chlozolinate, ketoconazole and linuron (Wolf et al., 1999; Makynen et al., 2000; Sohoni et al., 2001). The documentation about androgenic activity found in the aquatic environment relates mainly to pulp mill effluents that have been shown to have a masculinising effect on female mosquitofish (Howell and Denton 1989;

Cody and Bortone, 1997; Parks et al., 2001) with the causative agents being wood-derived AR ligands (Bortone and Cody, 1999). Sterols released from cooked wood pulp are the prime suspects. Bacteria in the water and river sediments convert the sterols to male androgen hormones, which then contaminate the water and influence fish development (Jenkins et al., 2001; 2003). Androgenic activity effecting fish has also been found in rivers downstream of U.S. beef-production facilities (Ankley et al., 2003; Durhan et al., 2006), where the anabolic steroid trenbolone acetate used as a growth promoter is hydrolysed to 17β-trenbolone which is a potent environmental androgen (Katsiadaki et al., 2007).

2.2. Endocrine regulation of reproduction in fish

Sexual differentiation of developing gonads in fish is under the control of hormones of the hypothalamo-pituitary-gonadal axis. Sex steroids play a critical role in early differentiation of the gonads into the two sexual types and also subsequent maintenance of the differentiated types (Devlin and Nagahama, 2002). The gonadotropin-releasing hormone is the primary hypothalamic neurohormone that stimulates the release of gonadotropins from the anterior pituitary. Both follicle-stimulating hormones and luteinizing hormone homologues exists in teleosts and are referred as to gonadotropin-I (GtH-I) and gonadotropin-II (GtH-II), respectively (Redding and Patiño, 1993; Nagahama, 1994). Gonadotropins stimulate gametogenesis and synthesis of gonadal sex steroids such as T and E2, which in turn provide feedback to the hypothalamo-pituitary axis to regulate sexual maturation and spawning. Steroid hormones are produced in the gonads; namely the ovaries of females and the testes of males.

2.2.1. Ovaries

In teleosts, the ovaries are paired structures attached to the body cavity. The follicle cell layer consists of an inner granulose cell layer, and outer sub layers of theca cells. Once the oocyte starts growing, the follicular layers change in order to support, nourish and regulate its development. The pituitary controls the release of GtH-I, which stimulates the production of sexual hormones by the theca cells, such as T and its aromatization to E2 in the granulose (Nagahama, 1994). In a response to the E2 stimulus, the liver produces vitellogenin (VTG), which is sequestered by the oocytes in a receptor-mediated process enhanced by GtH-I. Oocyte growth involves the uptake of the yolk protein precursor VTG from the plasma into the oocyte (van den Hurk and Peute, 1979). The oocyte development consists of several growth stages. The primary growth is characterized by substantial increase of the cell size, with a centrally located nucleus containing several nucleoli. The secondary growth period begins when prominent vesicles appear, a process that is known as endogenous vitellogenesis. The tertiary growth period, controlled by GtH-II, is known as the exogenous vitellogenesis. During this period the enlargement of

the oocyte is attributed to the accumulation of yolk and both the theca and granulose cells are thoroughly developed (Wallace, 1985). The mature oocyte becomes an extremely compact cell during ovulation. Atresia (degeneration) is common in the fish ovary and can occur at any developmental stage. Atresia involves apoptosis triggering and hormonal modelling (Jans and van der Kraak, 1997).

2.2.2. Testes

Androgenic steroids are produced by the testes and regulate male secondary sexual characters and sexual behaviour. In teleosts, testes are elongated-paired organs composed of branching seminiferous tubules or lobules and attached to the dorsal body wall. Spermatogenesis occurs within roundish cysts formed by Sertoli cells. Sertoli cells are found in direct association with germ cells, which they support physiologically and nurture. Features of the Sertoli cells suggest phagocytosis and an involvement in metabolic transport. The function of the Sertoli cells is equivalent to the ovarian granulosa cells. The Leydig cells are present in the connective tissue surrounding the lobules. Their function is the androgen synthesis needed for spermatogenesis and for expression of secondary characteristics, which is equivalent to theca cells in ovaries (Hoar and Nagahama, 1978; Redding and Patiño, 1993). Gonadotropin stimulates the secretion of fish androgen (11-ketotestosterone, 11-KT) from Leydig cells, which activates Sertoli cells to produce mediating factors that initiate the spermatogenesis. Initial cysts form by mitotic proliferation of spermatogonia, which develop into spermatocytes that undergo meiotic division producing secondary spermatocytes. A second meiotic division gives rise to spermatids, and differentiation of the spermatids form the spermatozoa (Nagahama, 1983). The duration of spermatogenesis is species specific. The mature sperm is finally released into a central lumen within the testis.

2.2.3. Steroid hormone biosynthesis

All steroid hormones are synthesised from the same cholesterol precursor via a series of biosynthetic steps catalyzed by different steroidogenic enzymes (Fig. 1). The end product depends on the complement of enzymes present in the tissues (Miller, 1988). The adrenal cortex is responsible for production of mineralocorticoids (aldosterone), which regulate the body’s levels of sodium and potassium. The adrenal cortex also produces glucocoricoids (cortisol), which regulates the carbohydrate metabolism. The Leydig cells in the testes synthesise and secrete the androgen testosterone. The theca cells in the ovaries synthesise and secrete androgens, while the granulose cells convert these androgens to estrogens.

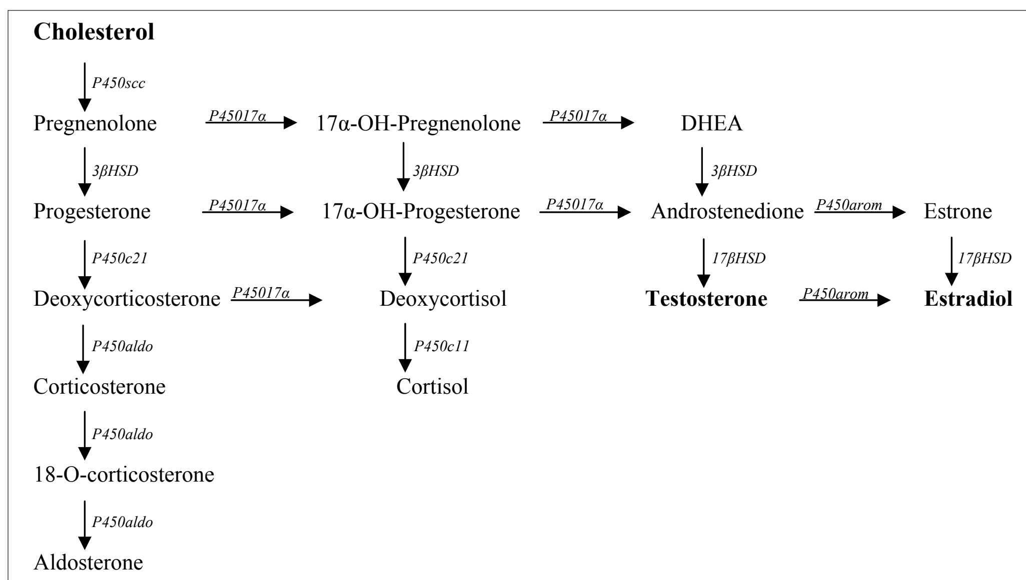

Figure 1. The steroid hormone biosynthesis pathway. Cholesterol is transformed through several enzymatic conversions to different steroid hormones.

The first rate-limiting step in the synthesis of steroids is the conversion of cholesterol to pregnenolone. The enzyme system that catalyzes this reaction is known as P450-linked side chain cleaving system (P450scc) and is found in the mitochondria of the steroid-producing cells. Pregnenolone may be converted to progesterone, or undergo hydroxylation to yield 17α-hydroxypregnenolone. Progesterone may also be hydroxylated, resulting in 17α-hydroxyprogesterone. The enzyme 21β-hydroxylase (P450c21) can hydroxylate these steroids resulting in deoxycorticosterone and deoxycortisol. The final step in the synthesis of the glucocorticoid cortisol is mediated by 11β-hydroxylase (P450c11). The enzyme aldosterone synthase (P450aldo) is responsible in converting corticosterone to aldosterone, the principal and most potent mineralcorticoid. 17α-hydroxypregnenolone and 17α-hydroxyprogesterone may also be converted to the androgens dehydroepiandrosterone (DHEA) and androstenedione. The conversion of androstenedione to testosterone is mediated by 17-hydroxysteroid dehydrogenase (17βHSD). Androgens are precursors for estrogens in the females and the aromatisation of estrogenic steroids from them is mediated by P450arom. T and E2 are carried in the plasma and delivered to target tissue by specific gonadal-steroid binding globulins (Miller, 1988).

2.3. Sewage treatment plants

Municipal wastewater includes residential, commercial and industrial liquid waste discharges. Today, the sewage treatment usually involves three main stages. *Primary treatment* allows the physical separation of solids and greases from the wastewater.

Secondary treatment is a biological treatment process where the wastewater is mixed with solids containing micro-organisms that use oxygen to consume the remaining organic matter in the wastewater as their food supply. Finally, during *tertiary treatment* the biological solids are neutralised, disposed of, and the treated water may be disinfected chemically or physically and the final effluent can be discharged. As the EDCs are not fully eliminated in the treatment processes, the effluent water will act as a carrier for their transportation to the environment. Some compounds are affected by degradation processes in the aquatic environment while some of the compounds pose a significant persistency. Effective effluent treatment at sewage treatment plants is of critical importance for preventing the contamination of the aquatic environment by EDCs. However, many potential endocrine disruptors exist as mixtures. Individual chemicals within these mixtures may vary greatly in potency and may interact with each other in an unpredictable manner. Sewage treatment plants may receive various amounts of these compounds depending on the nature of the local industry. The main abiotic factors that enhance degradation processes are elevated temperature, increased sunlight, and aerobic conditions. Degradation rates are therefore expected to be faster in warmer, sunnier parts of the world (IPCS, 2002). Since the steroid estrogens are biodegradable, it might be predicted that longer treatment time of the wastewater would lead to more removal of these compounds (Johnson et al., 2005). Increasing treatment time may be impractical for many sewage treatment plants. In lieu of increased treatment time, other tertiary treatment systems such as ultrafiltration, ozonation, UV treatment, and activated charcoal have been proposed (Johnson et al., 2005).

2.3.1. Reproductive effects of wastewater effluents in fish

The observations of hermaphrodite fish in sewage treatment lagoons in the UK during the 1970s and 1980s initiated an interest of EDC in effluents (Matthiessen and Sumpter, 1998). Estrogenic effects expressed as elevated levels of VTG in fish caged in receiving waters of sewage effluents in the UK was also reported by Purdom et al. (1994). These observations indicated that the observed disorders resulted from exposure to contaminants present in the river water, presumably originating from the sewage effluents. Since then, estrogenic disruption has been demonstrated in a number of fish species in receiving waters of sewage effluents in several countries. For example, high incidence of intersex characteristics (co-occurrence of ovarian and testicular tissue in gonads) has been observed in wild populations of roach (*Rutilus rutilus*), flounder (*Platichthys flesus*) and gudgeon (*Gobio gobio*) (Jobling et al., 1998; Allen et al., 1999; van Aerle et al., 2001, Kirby et al., 2004, Bjerregaard et al., 2006). Further, sewage effluent has been suggested to be the causal factor for elevated levels of VTG and inhibition of gonadal growth observed in feral male bream (*Abramis brama*) (Hecker et al., 2002; Vethaak et al., 2005) and depressed T levels in male carp (*Cyprinus carpio*) (Petrovic et al., 2002; Lavado et al., 2004). Environmental androgens and anti-androgens have until recently

been overlooked, most likely because of the lack of a sensitive system for the detection of androgenic activity (Katsiadaki et al., 2006). The clearest case of androgenic disruption in the aquatic environment is the masculinisation of fish living downstream of pulp mill effluent discharges (Howell et al., 1980). Androgens have, however, also been identified in sewage effluents with no secondary treatment (Thomas et al., 2004). Detection of androgenic compounds in domestic effluents has only been reported in a limited number of studies most likely due to successful disposal of androgens during secondary treatment at sewage treatment plants.

2.4. Biomarkers

Several sensitive biomarkers have been developed and applied as biomonitoring tools. Biomarkers have been defined as *"a change in biological response, which can be related to exposure or toxic effect of environmental chemicals"* (Peakall, 1994). Physiological biomarkers relate to biological responses at an organism level as suggested by Peakall (1999), and include measurements in the blood, organ-level measurements and indices, and integrated whole-animal responses (animal physiology). Exposure to environmental chemicals may also result in changes in the histological structure of cells and the occurrence of pathologies, which can significantly modify the function of tissues and organs. Evaluation of gonadal development has therefore successfully been used as a pathological biomarker in evaluation of sexual determination and differentiation.

2.4.1. Vitellogenin

VTG is a female-specific phospholipoglycoprotein of 200–700 kDa where the stickleback VTG is approximately 637 kDa and is a principal precursor of egg-yolk proteins crucial for successful embryonic and larval growth. VTG is synthesized in the liver of oviparous vertebrates, such as fish, in response to circulating estrogens and transported by the bloodstream to the ovary where it is taken up by oocytes, cleaved into the final egg-yolk proteins lipovitellin and phosvitin and deposited as yolk granules or platelets (Wallace, 1985). In vertebrates, plasma VTG dimers are sequestered via receptor-mediated endocytosis into the growing oocytes where further site-specific cleavages occur to yield smaller yolk proteins (Wahli, 1988). The VTG protein is secreted from the cell through the secretory pathway before it enters circulation and is taken up by the growing oocyte. This makes blood plasma a natural target for VTG analysis. Plasma VTG concentrations are normally an indication of the maturational status of the female fish (reviewed in Mommsen and Walsh, 1988; Arukwe and Goksøyr, 2003). Several studies demonstrated that even male fish caught in rivers and streams had high levels of plasma VTG (Purdom et al., 1994; Jobling et al., 1998), caused by chemicals acting like estrogens present in the environment. VTG induction in fish has become an accepted measure of xenoestrogenic potency of chemicals, effluents and discharges.

2.4.2. Spiggin

The androgen-induced glue protein used for nest building is only produced in the kidneys of male three-spined stickleback during the breeding season. The glue was characterised as a 203 kDa glycoprotein by a group in Sweden and was called spiggin after *spigg,* the Swedish name for the three-spined stickleback (Jakobsson et al., 1999). Spiggin is very hydrophobic and was found to be a novel protein with structure similarities to von Willebrand Factor (Jones et al., 2001). There is a whole family of genes encoding different spiggin types (Kawahara and Nishida, 2006). The half-life of spiggin is not yet determined but Katsiadaki et al. (2007) suggest that spiggin from a fully hypertrophied kidney requires at least one month to regress to the point where it is not detectable. Female sticklebacks do not produce spiggin under normal conditions, however, spiggin production in females was observed when exposed to androgens in laboratory conditions (Katsiadaki et al., 2002). Spiggin is to date the only known androgen-induced protein in fish and is therefore considered as a potential androgen biomarker.

2.4.3. Circulating hormones

Circulating levels of hormones are usually determined in plasma samples. The effect of changes in circulating levels of the sex hormones has been a subject of research in endocrine disruption. A decrease in sex steroid concentrations in response to EDC exposure is reported, even though the mechanism of action is not known (Sumpter, 1997; Tyler et al., 1998; Snyder et al., 2004).

2.4.4. Condition indices

Physical condition indices have been used for many years as a simple method for monitoring changes in fish health. The most common morphometric index is the condition factor. Organosomatic indices (ratios of organ mass to body mass) may, however, provide more specific information relating to the function of the selected organs. Condition indices are influenced by environmental factors such as season and temperature and the physiological status of the animal such as nutrition (Russel et al., 1996; Khallaf et al., 2003). Temporal changes may therefore not be directly related to pollutant exposures. On the other hand, pollutants can produce rapid and marked changes in condition indices clearly differentiated from any seasonal or life-cycle influence (Jobling et al., 1996).

2.5. Screening of endocrine disruption in fish

In the context of pollution, aquatic systems are highly vulnerable due to their tendency to accumulate relatively high concentrations of chemicals. Fish therefore provide a useful model for the assessment of environmental pollutants, and also in terms of species extrapolation. General test methods used by fish toxicologists originate from the

1800s and were adapted from techniques used in mammalian toxicology. Goldfish and minnows were the first fish species used in aquatic toxicity tests to determine the effects of chemicals used in dye-works (Penny and Adams, 1863). It was not until prior to World War II that fish acute toxicity studies became a reality in testing industrial wastes and metals (Hart et al., 1945). Toxicity tests are generally conducted to assess or predict the biological effects of chemicals. Just as there are many different biological levels of organization that a chemical can exert effects, a variety of methods are available.

Both *in vivo* and *in vitro* bioassays have been developed for detection of chemicals and effluents with endocrine disrupting properties. Regulatory *in vivo* screening and testing programs incorporate assays with usually small freshwater fish species (Ankley and Johnson, 2004). The advantages are their relatively small size and short life cycle facilitating both short-term tests (typically for 21 days) and large-scale experiments of partial or full life cycle toxicity testing. Fish *in vivo* assays have been developed for EDC testing with endpoints at multiple levels of biological organization. Measurements include endpoints such as survival, growth, morphological development and reproduction, secondary sexual characteristics, plasma steroids, VTG and gonad histology (Ankley and Johnson 2004). *In vivo* testing is cost-prohibitive and often discouraged due to the large number of animals needed. Therefore, several *in vitro* screening tests have been developed. The major biological methods available for detecting hormonally active substances are *in vitro* bioassays for assessing estrogenic or anti-estrogenic substances include subcellular hormone receptor ligand-binding assays, hormone sensitive transcription of reporter genes, steroidogenesis assays, hormone responsive mammalian cell proliferation assays and assays measuring estrogen-responsive gene expression or protein synthesis in cell cultures (ICCVAM and NICEATM, 2002). *In vitro* fish screening systems have been developed for the assessment of chemical biotransformation or metabolism by using tissue slices, perfused tissues, fish embryos, primary and immortalised cell lines, and sub-cellular fractions (Weisbrod et al., 2008). Some estrogenic and anti-estrogenic endpoints, such as VTG production, can be measured using piscine *in vitro* systems, in particular by primary cultures of hepatocytes (Jobling and Sumpter, 1993; Pelissero et al., 1993; Anderson et al., 1996; Gagné and Blaise, 1998; Segner et al., 2003). The main advantage of the hepatocyte VTG assay is considered its ability to detect effects of estrogenic metabolites, since hepatocytes *in vitro* remain metabolically competent (Navas and Segner, 2006). The examination of androgenic effects *in vitro*, using fish cells has not been assessed sufficiently. Instead, different human cell lines containing the androgen receptors or bioluminescent yeast-based bioassay for androgen-like compounds have been used (Terouanne et al., 2000; Wilson et al., 2002; Paris et al., 2002; Michelini et al., 2005; Sonneveld et al., 2005). It should be noted that *in vitro* assays may not perfectly reflect the true *in vivo* response in fish to the same compound. *In vitro* assays have several disadvantages, including the inability to account for bioaccumulation and

lack of metabolic capacity. However, *in vitro* models enable studies on the mechanism of action and can facilitate data in a time and cost effective manner. Methods that rely on biological activity are finding increased utility, particularly as screening tools, because the chemical nature of a sample may not be known and a biological activity may be the best, or only, indicator of EDCs. A combination measurement of both chemical and biological activity is often desirable.

2.6. The three-spined stickleback as model system in toxicological studies

The three-spined stickleback is a small teleost species with an exceptionally wide geographical distribution. The three-spined stickleback's origin lies within the marine environment, but it is also present in brackish and freshwater environments (Curry-Lindahl, 1985). Sticklebacks breed once a year starting in early spring. After the male establishes a territory, he builds a nest, using the sticky protein spiggin produced in the kidney, and develops nuptial coloration such as red throat and blue irises. The role of androgens in stimulating reproductive behaviour and secondary sexual characters has been extensively studied in sticklebacks (Borg and Mayer, 1995; Mayer et al., 2004). The completion of the full genome sequencing (estimated genome of 675 mega-bases) will also give this species a clear advantage over other models (Katsiadaki et al., 2007). The stickleback also has unique markers for detection of endocrine disrupting chemicals including the estrogenic biomarker VTG, the specific androgen endpoint spiggin, and the presence of a rudimentary Y chromosome for assignment of a genetic sex (Griffiths et al., 2000; Peichel et al., 2004).

The Organisation for Economic Co-operation and Development (OECD) is currently in the process of validating a short-term fish screening protocol for endocrine disrupters (estrogens, androgens and aromatase inhibitors) using three core species: the fathead minnow (*Pimephales promelas*) the zebra fish (*Danio rerio*) and the medaka (*Oryzias latipes*) (OECD, 1999). The main endpoints proposed for the first phase of validation of the screening are VTG induction, gross morphology (secondary sexual characteristics and gonadosomatic index) and gonadal histopathology. In view of the absence of all these species in the European environment, a similar protocol is concurrently being developed using the three-spined stickleback with the same endpoints but with the addition of spiggin induction as a superior androgen-specific endpoint (Katsiadaki et al., 2007). The researchers working with this are hoping that results will continue to demonstrate the potential of the stickleback as a test species and these results will facilitate the stickleback's inclusion into the final OECD Technical Guidelines.

3. OBJECTIVES

The objective of this study was to investigate endocrine disruption related to the release of anthropogenic (estrogenic and/or androgenic) substances via municipal wastewater effluents. One goal was to evaluate the usefulness of adult three-spined stickleback as model organism in the simultaneous assessment for estrogenic and androgenic modes of endocrine action in both field and laboratory based exposure studies. Another goal was to develop rapid *in vitro* screening techniques in order to simultaneously assess estrogenic and androgenic activity of chemicals and environmental samples, and to compare endocrine responses *in vivo* and *in vitro*. The following questions were addressed:

- Is estrogenic or androgenic disruption present among fish along the Finnish Baltic Sea coast in areas where sewage treatment plants discharge effluents?
- Is effluent from domestic sewage treatment plants estrogenic and/or androgenic to fish?
- What biomarkers are suitable and reliable in the development of validated protocols for the simultaneous assessment of estrogenic and androgenic impact?
- Is it possible to predict endocrine effects of complex environmental samples such as municipal wastewater with *in vitro* assays based on cell and tissue cultures?
- What substances are responsible for the observed effects seen *in vivo* and *in vitro*?

4. MATERIALS AND METHODS

The used materials and methods are briefly described in this chapter. Additional information can be found in the original publications and manuscripts.

4. 1. Experimental fish

4.1.1. Field sampling (I)

Adult three-spined sticklebacks were collected with seine or drop nets from six different sites along the Finnish coastline in the Baltic Sea. Four of the sites were known to be receiving sewage treatment effluents and two sites were considered reference sites located in an undisturbed environment (Fig. 1, I). A quota of 25-30 fish/sex was sampled from each site resulting in a total of 350 fish, and all fish collections were carried out in June during the time of natural spawning. A more targeted investigation was conducted at a contaminated site the following year with a non-contaminated reference site included. Fish (10-15 fish/sex) were collected during a whole reproductive period (May-August) to characterise seasonal variation in the reproductive parameters chosen to detect possible reproductive disturbances. All sewage treatment plants included in this study used mechanical, biological and chemical treatment processes and the total effluent discharge at each plant ranged from 210 000 to 6 700 000 m^3/year.

4.1.2. Fish collection and maintenance (II, III, IV)

Adult three-spined sticklebacks for the exposure experiments and *in vitro* experiments were caught with a seine or drop nets from an undisturbed environment in the south-western part of the Finnish coast in the in the northern Baltic Sea. Following transport to the laboratory, the fish were given a formalin bath (diluted 1:5000 in 3‰ brackish water, 5 minutes at room temperature) to remove possible ectoparasites. The fish were kept in 200 liter aquariums with artificial brackish water (3‰, Meersalz Proffessional), pH 7, at a short photoperiod of 8 h light: 16 h darkness at 12 °C. The fish were fed daily with frozen red midge larvae (*Chironomus* sp.) (Imazo Ab). Cells and tissues for the *in vitro* experiments were isolated from fish held at short photoperiod (quiescent fish) but also from fish that were photoperiodically stimulated. These fish were transferred from the short photoperiod into a long photoperiod of 16 hour light: 8 hours darkness at 18 °C for two weeks before being used. Only fish without signs of parasitism were used in all experiments.

4.1.3. Laboratory exposure conditions (II)

The fish were kept in 45 litre aquariums filled with artificial brackish water (3‰ salinity, HW Sea Salt Professional) pH 7, at 20 °C and at a photoperiod of 16 hour light: 8 hour dark. The fish were randomly separated into 12 groups consisting of 16 females and 16 males per aquarium. The aquaria were cleaned daily and water exchange was regulated by a continuous-flow through system allowing the total volume in the aquaria to be exchanged daily. The fish were acclimatised to the aquaria condition for 21 days prior to onset of the experiment. The fish were then exposed to 20 ng/l 17α-ethynylestradiol (min 98%, Sigma-Aldrich), 10 µg/l 17α-methyltestosterone (MT, min 97%, Acros Organics), wastewater effluent from the Turku sewage treatment plant in three concentrations (10%, 50% and 80%), and brackish water (negative control) in a continuous flow-through system, and the exposures were done in two replicates. The test chemicals were dissolved in 96% ethanol and the final concentration of the solvent did not exceed 0.00001%. After one week of exposure, half of the female and male fish were removed and the rest were exposed for an additional three weeks. Water temperature was measured daily and was 19-20 ºC over the period of the experiment. Water pH was measured every second day and was 6.9-7.4 over the period of the experiment.

4.2. Sample preparations and analysis

4.2.1. Fish sample preparations (I, II)

The fish from the field study and exposure experiment were sacrificed with an overdose of benzocaine (Oriola), measured for total length and weight to the nearest 0.01 g and a macroscopic evaluation for presence of parasites was done. Blood samples were collected from the caudal vein by severing the tail fin with a razor blade and collecting the blood in heparinised capillary tubes. The plasma was separated by centrifugation at 9000 x g for 2 minutes at 4 ºC and aprotinin was added (2 TIU/ml, Sigma-Aldrich). The samples were stored at –70 ºC prior to measurement of VTG and plasma steroids. Liver and kidney were excised, weighed and the kidney was snap frozen. The frozen samples were stored at –70 ºC until analyzed. Gonads were excised, weighed and fixed in 4% phosphate-buffered formalin for histopathological examination.

4.2.2. Morphological analysis (I, II)

The condition factor (CF) was calculated for individual fish as the ratio of ([body weight (g) – gonad weight (g)] / [total length (mm)]^3) x 100. The gonadosomatic index (GSI, the gonad weight as a percentage of body weight) was calculated for individual fish as the ratio of [gonad weight / (body weight – gonad weight)] x 100. The hepatosomatic index (HSI, the liver weight as a percentage of body weight) was calculated as the ratio of [liver weight / (body weight – gonad weight)] x 100. The nephrosomatic index (NSI,

the kidney weight as a percentage of body weight) was calculated as the ratio of [kidney weight / (body weight – gonad weight)] x 100.

4.2.3. Analysis of sex steroids (II)

E2 and T content of plasma were quantified using an E2 or T enzyme immunoassay, respectively, as described by the manufacturer (Cayman Chemical Company). The plasma samples were diluted 1:50 in coating buffer belonging to the assay and all samples were assayed in duplicates. In the calculation of steroid concentration the values were corrected for the amount of aprotinin added.

4.2.4. Histological assessment of the gonads (I, II)

Gonads fixed in 4% phosphate-buffered formalin were processed for histological examination by light microscopy (Fig. 2). After fixation, the tissues were rinsed with water, dehydrated through a series of graded ethanol solutions (30-99%), cleared in xylene, and embedded in paraffin in an automatic tissue processing (Histokinette 2000, Reichert-Jung). Tissue sections of 5 µm were cut and the sections were stained in Delafield's haematoxylin (2%) and eosin (0.5%). Four follicular stages were identified and counted from the ovaries; primary oocytes, secondary oocytes (yolk vesicles present), mature tertiary oocytes (follicle boarded by an egg membrane) and atretic (degenerated) oocytes. Granulomatous inflammatory reactions or other malformations were also observed. Histological alterations in the testes were evaluated based on the presence of different stages of sperm cells, increased fibriosis, presence of phagocytising Sertoli cells and the general lobular structures. Representative light micrographs were taken with a light microscope (Leica TCS 4D) coupled to a CCD camera. Analysis of histopathological changes in the gonads was always performed by visual scoring of coded slides (blind reading).

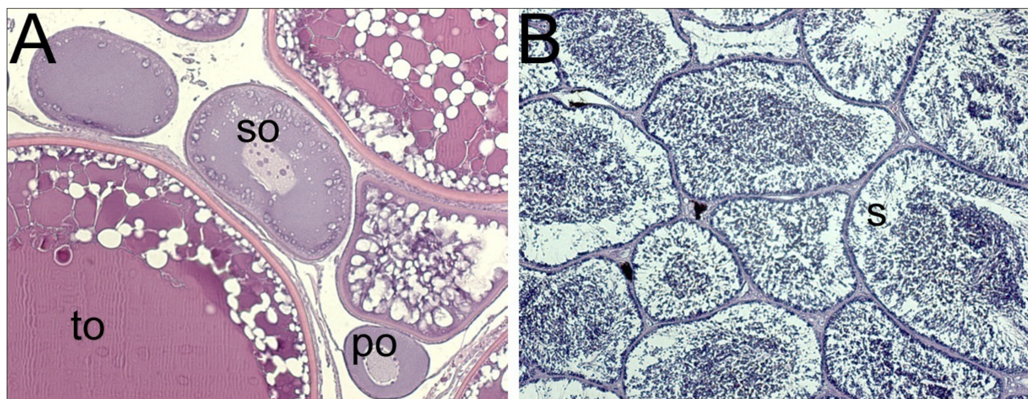

Figure 2. A. Presence of different oocytes in mature three-spined stickleback ovary. **B**. Sperm cells in mature stickleback testes. Abbreviations used: po – primary oocyte, so – secondary oocyte, to – tertiary oocyte, s – mature sperm.

4.2.5. Analysis of vitellogenin (I, II, III, IV)

The VTG content was analysed from the plasma of fish or from the cell media in the primary hepatocyte cell and liver tissue slice cultures. The content was quantified using a competitive ELISA and stickleback specific VTG antibodies as described by Katsiadaki et al., 2002 and Hahlbeck et al., 2004. VTG content was expressed as µg VTG/ml plasma with values corrected for the amount of aprotinin added. VTG levels from the cell cultures were expressed as % VTG of the total protein content (µg/ml). The liver tissue slices were weighted and VTG content from the liver tissue slice cultures was expressed as VTG/liver weight.

4.2.6. Analysis of spiggin (I, II, III, IV)

The spiggin content of the kidneys was quantified using an indirect ELISA and stickleback specific spiggin antibodies. The kidneys were dissolved in 200 µl denaturing buffer (100 mM Tris-HCl pH 8.5, 10 mM EDTA, 8 M Urea, 2% SDS, 200 mM β-mercaptoethanol) and heated at 80 °C for 1 hour. The spiggin content produced by the kidney cell cultures accumulated inside the cells and had to be extracted trough a procedure of repetitive snap freezing and thawing. Samples were diluted in 0.05 M sodium bicarbonate-carbonate buffer pH 9.6 and coated onto a microtiter plate (Polysorp, Nunc) over night. Blocking of unspecific binding was performed with 1% milk powder solution diluted in PBS, for one hour at room temperature. The plates were washed three times with washing buffer (PBS with 0.05% Tween 20) before incubation for one hour at 37 ºC with a specific rabbit polyclonal stickleback spiggin antibody diluted 1:3000 in PBS containing 0.05% Tween 20. The polyclonal spiggin antiserum was raised against an internal peptide (HRDELIRDSKLHDHRC) corresponding to amino acids 173 to 188 (Agri Sera AB, Umeå, Sweden). After washing, the plates were incubated with an alkaline phosphatase conjugated anti-rabbit IgG whole molecule (Sigma-Aldrich) diluted 1:15000 in PBS containing 0.05% Tween-20 for one hour at room temperature. The plates were washed and 1 mg/ml p-nitrophenyl phosphate (Sigma-Aldrich) in 0.2 M Tris buffer was added and the plates were then incubated in room temperature protected from light during 30 minutes. The microtiter plates were spectrophotometrically measured at 405 nm with a Victor^2 1420 multilabel counter (Wallac, PerkinElmer). Spiggin content was expressed as spiggin arbitrary units (A.u/mg), which were calculated as the ratio of the sample absorbance / kidney weight (mg). Spiggin levels from the primary cell cultures were expressed as relative absorbance values in ratio to the total protein content.

4.3. Wastewater collection (II, III, IV)

The municipal sewage treatment plant in Turku processes domestic and industrial wastewaters from a population of 160 000 people and utilizes chemical and biological

treatment processes. The influent flow is approximately 60,000 m^3/day and the Baltic Sea is the recipient of the plant's final effluent. Wastewater effluent was collected every second day during the *in vivo* exposure experiment in summer 2005 in high-density polyethylene cans. The crude wastewater was filtered through a mesh (100 µm) and made brackish (3‰ salinity) before being added to the aquariums as 10%, 50% or 80% dilutions of original wastewater. Twenty-four hours composite effluent samples were collected in summer and autumn 2005 in glass bottles for chemical analysis. The samples were divided into smaller portions for the different preparations and the processing of the samples at the laboratory started immediately after collection to minimize possible degradation of the samples.

4.4. Cell and tissue culturing

4.4.1. Primary hepatocyte cultures (III, IV)

The fish were sacrificed as previously and the livers were immediately excised and transferred to phosphate buffered saline (PBS) containing 50 µg/ml gentamicin (Gibco Invitrogen) on ice for an hour. The livers were pooled together, rinsed with PBS containing 25 mM Tricine and 0.5 mM EGTA, and digested with digestion buffer (PBS, 0.05 M HEPES, 0.05 mM $CaCl_2$) including collagenase type IV (0.25 mg/ml, Sigma-Aldrich) during 20 minutes in room temperature on a shaker (200 rpm). The cell suspension was filtered through a 100 µm and a 40 µM nylon mesh (Primaria cell strainer, BD). The cell medium consisted of phenol red-free Leibovitz´s L-15 medium (Gibco Invitrogen), 1 µg/ml fungizione (Gibco Invitrogen), 50 µg/ml gentamicin (Gibco Invitrogen), 100 U/ml penicillin-streptomycin (Gibco Invitrogen), 15 mM HEPES (Cambrex BioScience) and 5% heat-inactivated foetal calf serum (FCS, Gibco Invitrogen). The cells were washed and counted in a Bürker chamber. Viability of the cells was >90% as assessed with the trypan blue exclusion test. Cells were plated at a density of 300,000 cells/well in white, clear bottom Primaria 96-well microtiter plates (BD) and cultured at 18 °C in an incubator (Termaks).

4.4.2. Primary kidney cells cultures (III)

The fish were sacrificed as previously and the posterior parts of the kidneys were immediately excised and transferred into PBS containing 50 µg/ml gentamicin on ice for an hour. The pooled kidneys were treated with 0.05% trypsin solution containing 0.02% EDTA (Gibco Invitrogen) for 30 minutes at room temperature on a shaker (200 rpm). The suspension was filtered through a 70 µm nylon mesh (Primaria cell strainer, BD). The cell medium used for culturing the kidney cells was the same as described for culturing hepatocytes. The cells were washed, counted in a Bürker chamber, and the concentration was adjusted to 1.0 – 1.5 x 10^6 cells/ml for females, and 0.50 – 0.75 x

10^6 cells/ml for males. Viability of the cells was >90% as assessed with the trypan blue exclusion test. The kidney cells were cultured in Primaria 96-well microtiter plates (BD) at 18 °C in an incubator.

4.4.3. Primary tissue slice cultures (III)

Livers and kidneys, respectively, were not pooled in the tissue slice cultures, rather, it was of importance to obtain control tissue and treated sample tissue from the same donor animal. Excised livers and kidneys were separately transferred into PBS containing 50 µg/ml gentamicin on ice for an hour. The tissues were cut into smaller pieces with a razor blade and transferred into a 48 well microtiter plate (Nunc) containing 500 µl/well of cell culture media including FCS, as used in the cell culture assays. The tissues were cultured with shaking (100 rpm) at 18 ºC in an incubator.

4.4.4. Cell culture exposures (III, IV)

The isolated cell cultures were cultured as a monolayer for 24 h in cell medium containing FCS allowing cell attachment to the microtiter plate. The cells were gently washed three times with 1x PBS before *in vitro* treatments. During the exposure the cells were cultivated at 18 °C in an incubator. The incubation of hepatocytes was terminated after 72 h by carefully collecting all cell medium and storing the medium samples at –20 ºC until further processing. The incubation of kidney cells was terminated after 72 h by lysing the cells through repeated freeze-thaw cycles of the microtiter plates. The tissue slice cultures were cultured for 24 h and washed three times with PBS before the *in vitro* treatment. The incubation of the tissue slice cultures was terminated after 24 h by collecting the media from each well and storing the samples at –20 ºC until further processing. Stock solutions of hormones (10 mM) were prepared in ethanol and stored at –20 ºC. Working solutions were prepared on the day of use in cell culture media without FCS. Final concentration of the solvent in the tests did not exceed 0.1% but solvent controls were included.

Estrogens: Tested estrogenic hormones were E2 (min 98% purity, Sigma) and 17α-ethynylestradiol (EE2, 98% purity, Sigma-Aldrich) at concentrations of 10 nM-10 µM.

Androgens: Tested androgen hormones were MT (min 97% purity, Acros Organics), 11-ketotestosterone (11-KT, Massey University) and 5α-dihydrotestosterone (DHT, min 99% purity, Sigma-Aldrich) at concentrations of 10 nM-10 µM.

Wastewater effluent: Solid phase extractions (SPE) were dissolved in 10 ml serum-free cell culture media and the primary cell and tissue slice cultures were exposed to 10%, 50% or 80% of the original wastewater. Chromatographic fractionations of the SPE extracts were also analysed in three different concentrations, 10%, 50% and 80% of

original wastewater sample, and the *in vitro* treatment of the chromatographic fractions was performed by coded samples (blind test).

4.4.5. Total protein measurements (III, IV)

The total protein content of the primary cell cultures was determined using the Quick Start Bradford Protein Assay (BioRad) as described by the manufacturer and the absorbance was measured spectrophotometrically at 595 nm with a Victor^2 1420 multilabel counter. An external bovine serum albumin standard was included for calculation of total protein content. Concentrations of VTG and spiggin were normalised total protein content.

4.4.6. Viability assay (III, IV)

The viability of the cell cultures were analysed with the viability marker calcein-AM (Molecular Probes). Calcein-AM (6 µg/ml) and HEPES-Cortland buffer (100 µl) was added to the cells, incubated 20 minutes at room temperature and the fluorescence was measured with a Victor2 1420 multilabel counter at the excitations wavelength of 485 nm and emission wavelength of 535 nm. The amount of living cells in the treatments was calculated based on fluorescence values in comparison to the controls (non-treated cells).

4.4.7. Immunocytochemistry (III)

For immunocytochemical characterisation of spiggin producing cells, cells isolated from stickleback kidneys were cultivated at a density of 0.5 x 10^6 cells/ml for 24 h on sterile Lab-Tek chamber slides (Nunc International) pre-coated with gelatine and chromium potassium sulphate. The cells were *in vitro* treated with MT or DHT for an additional 72 hour. The cells were washed three times with ice-cold PBS before fixation with 4% PFA solution for 20 min at room temperature. The staining procedure was done with the Vectastain ABC kit (Vector Laboratories) and 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich) in accordance to the manufacturers guidelines. Primary antibody was the same spiggin antibody as the one used for ELISA and this was incubated at one hour at room temperature diluted 1:5000 in PBS.

4.5. Chemical analysis (II, III, IV)

Solid phase extraction (SPE) was used for isolation and concentration of the organic constituents of the wastewater effluent. Samples of 100 ml of the effluent water were passed over Oasis HBL-columns (Waters, 200 mg, 6 cc) using the procedure described by Salste et al., 2007. The analysis of estrogens in the wastewater effluent, and analysis of the actual concentration of EE2 in the exposure experiments was performed with an optimised LC-MS/MS method reported previously (Salste et al., 2007). For analysis

of the actual concentration of MT in the exposure experiment, the water samples were extracted by liquid-liquid extraction with MTBE at pH 3. An amount of 2 ml of MTBE-solution (0.02 mg/ml) containing the internal standards was added to the extraction. Furthermore, the sample was dried in a vacuum oven for 20 minutes before silylation. The samples were transferred to GC vials and the extracts were analyzed by GC-FID with a HP-1 column (25 m, 0.20 mm i.d.) for the individual components. GC-MS analysis was used for verification of MT in the GC peak. The chromatographic fractionation of wastewater effluent was performed on an Agilent 1100 Series liquid chromatographic system as described elsewhere (Salste et al., 2007).

4.6. Statistics

In order to evaluate statistical significances, a one-way ANOVA followed by the Bonferroni or Fisher´s post hoc test was performed. If necessary, values were transformed (square root, $\log_{10}$) when needed for homogeneity of variance. The parameters measured from different sampling sites during different sampling periods were analysed with two-way ANOVA. Kruskal-Wallis test was used if normality or homogeneity tests failed. Correlations coefficients between biomarkers were calculated using the Pearson or Spearman´s tests. All calculations were made with the SPSS 14.0 (SPSS Inc. Chicago).

5. RESULTS AND DISCUSSION

5.1. The three-spined stickleback as model species in bio monitoring (I)

The purpose of this study was to examine the impact of sewage-derived endocrine disrupters (estrogens and androgens) on fish in the Baltic Sea. Another purpose was to assess the suitability of different reproductive biomarkers present in three-spined stickleback in the detection of endocrine disruption in the aquatic environment.

5.1.1. Morphometric analysis

The condition factor (CF) relates body length to weight. The condition factor is widely used in fish biology to provide an easily assimilated descriptor of health and well-being. Stress decreases the condition factor, which is often interpreted as a decline in body fat or stored glycogen in the liver. Pollutants that cause an increase in metabolic rate, a decline in energy uptake, or enhance fat metabolism as a part of toxic action are likely to decrease the condition factor (Smolders et al., 2003). It may not always be possible to elucidate pollutant effects on condition factors because the effects of pollutants are masked by other biotic or abiotic variables. The condition index may be used as a good comparison of populations to establish whether there is evidence of greater accumulation of body mass in one or other population. The sampling method in the current study was, to some extent, selective since the mesh size of the nets used was such that the smallest fish present were not retained. The condition factor was not affected in fish sampled from different sites in the field study except at one site next to a sewage treatment plant, where both males and females exhibited lower CF compared to reference fish suggesting poorer conditions (Site F, Table 2, I). No variation in CF was observed for sticklebacks sampled at different time periods (Table 4, I), which is in agreement with other studies (Roussel et al., 2007; Sanchez et al., 2008).

The hepatosomatic index (HSI) is one of the most common applied indices because of the central role of the liver in the detoxification of pollutants. Goede and Barton (1990), however, pointed out that the HSI can decline in response to starvation, but liver weight may increase due to pathological changes (e.g. hyperplasia associated with pollutant exposure). It is important to remember that an increase in HSI also normally occurs in female fish during vitellogenesis (van Bohemen, 1981). In the field study there were variations in the HSI in females caught at the different sites. Generally, females sampled from the contaminated sites showed an increase in the HSI (Table 2, I). However, the females showed decreased HSI at site F. Sublethal levels of crude oil have been shown to cause a significant decrease in HSI in striped mullet (*Mugil cephalus)* (Chambers 1979) and HSI also decreased significantly in brown trout (*Salmo trutta*) and rainbow

trout (*Oncorhynchus mykiss*) that were subjected to aquatic environments with low pH (Jacobsen, 1977; Lee et al., 1983). Since the sampling site F receives both sewage water effluents and is a harbour area with daily ferry traffic, the decreased HSI could be due to pollutants from the ferry traffic in combination with impaired water quality.

The gonadosomatic index (GSI) is used in many studies to assess the impact of EDCs on the reproductive system. GSI decreases have been reported in adult rainbow trout and carp exposed to estrogens either in water or via their food (Komen et al., 1989; Jobling et al., 1996; Gimeno et al., 1998). The GSI is also considered suitable for detection of estrogen mimics (Jobling et al., 1996). A decrease in GSI was also observed in this study in male and female fish caught from site F, suggesting possible estrogenic exposure at the site (Table 2, I). However, there was high variability in GSI in the sampled fish showing different maturation stages at the different sampling sites.

Compounds with estrogenic activity have previously been shown to inhibit kidney growth (Katsiadaki et al., 2006; Andersson et al., 2007) and since a decrease in the nephrosomatic index (NSI) in males from site F was observed (Table 2, I), one explanation could be due to exposure to estrogenic compounds. However, the increase in NSI observed in females from the same site suggests the opposite, i.e. an androgen exposure. This inconsistency was solved when looking at NSI values during a whole reproduction period and similar NSI values was gathered, but at different time periods (Table 4, I). It is therefore very important to take the time period into account when using the NSI as a biomarker in sticklebacks in field monitoring.

5.1.2. Vitellogenin and spiggin

Levels of the estrogen-dependent yolk precursor VTG were not strongly elevated in male sticklebacks and VTG were not present in males to the same extent as female VTG levels (µg/ml compared to mg/ml respectively). However, VTG was detected in male sticklebacks from the contaminated sites, reflecting a possible exposure to estrogenic compounds in the environment (Table 3, I). There was an overall significant difference in VTG concentrations in males between the sites. VTG content in males from the contaminated sites and especially from site F (6.3±1.5 µg/ml) was significantly higher than VTG content in males from the reference sites (mean values 1.3±1.0 µg/ml). Contradictory to these results, females from site F had significantly lower levels of VTG (1.0 mg/ml) compared to the reference sites (6.8 mg/ml), while females sampled from the other sites next to the sewage treatment plants had elevated levels (in average 11.7 mg/ml) as the males sampled from the same contaminated sites. Some of the variation in VTG concentration in exposed male and female fish in the wild is caused by differences in the timing and duration of their exposure to estrogenic compounds (Jobling et al., 2006), since VTG has a half-life in plasma from 10-21 days (Schmid et al., 2002). Consequently, VTG is not a stable biomarker of long-term exposure to estrogens and it

is much more likely to be sensitive to changes in short-term temporal factors related to changes in water flow. It was confirmed that males sampled from the contaminated site F showed significantly increased VTG levels compared to the reference site when VTG was analysed during an entire breeding season (Table 5, I). The results are therefore suggesting exposure to estrogenic compounds. The lower VTG content in females observed at site F was shown to be the case also during a whole reproductive period, however, the peak of maximum amount of VTG was the same as at the reference site, but then the VTG concentrations decreased very rapidly to significantly lower levels compared to reference levels, suggesting also the presence of some anti-estrogenic substances at that site.

The induction of the androgen-dependent protein spiggin in females sampled from the contaminated sites D (17.5±4.4 A.u/mg) and E (18.5±4.0 A.u/mg) compared to fish sampled from the reference sites (average 10.5 A.u/mg) suggests that these fish could have been exposed to androgenic compounds (Table 3, I). Recently, Blankvoort et al. (2005) reported contamination of European rivers by androgens including testosterone and its metabolites and 17α-methyltestosterone that were released into aquatic ecosystems by sewage treatment plants and industrial activities. Compounds like these could be capable of inducing spiggin levels in female sticklebacks, however, the induction was not very significant. Spiggin levels in males sampled from the different sites showed no increase in spiggin levels at the different sampling sites. To the contrary a decrease in spiggin levels was observed in males sampled from site F (48.2±22.5 A.u/mg) compared to reference sites having spiggin levels around 175 A.u/mg, suggesting the presence of anti-androgenic compounds at that site. When spiggin levels in fish from site F were further analysed during an entire breeding season (Table 5, I), it was shown that the males sampled from site F had reduced spiggin levels compared to the reference site but the levels increased during the sampling period to the same levels as the reference fish. The sampling time seems therefore to be of great importance when measuring spiggin content in male stickleback.

5.1.3. Histopathology

Histopathology has proven to be a useful biomarker to evaluate the potential risk to aquatic organisms posed by exposure to both natural and anthropogenic chemicals that may interfere with reproduction and development (Allen et al., 1999; Bateman et al., 2004). The gonads can be considered the primary organ targeted by exogenous compounds with estrogenic or androgenic activity. The maturation of ovaries in female fish inhabiting effluent-contaminated waters appeared to be less obviously affected, although a higher incidence of oocyte atresia was found in the fish sampled from the contaminated sites compared to fish sampled from the reference sites (Fig. 2, I). Increased atresia has

previously also been found in fish sampled from contaminated waters (Cross and Hose, 1988; Johnson et al., 1988; Jobling et al., 2002).

The histological examination showed that the majority of the fish sampled from the contaminated sites had degenerated testes in which the normal lobular arrangements were disrupted and spermatogenesis was impaired. Accumulations of yellowish-brown pigmented cells in the lumen of the lobules were also observed in these testes. Male fish, especially those sampled from site F, showed severe changes, such as disorganisation of the lobules, inhibition of spermatogenesis, and increased necrosis compared to testes from the reference site (Fig. 3, I). The changes were observed in some of the testes already from the first sampling in May, and increased rapidly to include all examined testes sampled in July and August. Testes from fish sampled at the non-contaminated locations were in a mature stage containing mostly mature sperm and the connective tissue was thin, as were the interstitial compartments. No intersex fish were found in the field survey. In conclusion, there were clear changes in male testis development in the fish sampled from contaminated sites even though there was no occurrence of intersex. However, the severe changes seen in the male testis sampled from the contaminated sites gives no evidence if reproduction was disrupted in the populations. At least male roach (*Rutilus rutilus*) with mild intersex characteristics were recently shown to be able to compete with normal males and contribute to the next generation in a competitive breeding scenario (Matthiessen et al., 2008).

5.1.4. Parasites

Parasite infections influenced the reproductive parameters of the sticklebacks in the field study more than expected. The presence of *Schistocephalus solidus* and *Glugea anomala* were very common, especially in fish sampled from the contaminated sites, but these parasites were also present in fish sampled from reference sites. Other parasites such as *Gyrodactulus* sp., *Trichodina* sp., *Argulus* sp., *Raphidascaris* sp., *Thersitina gasterostei* and *Triaenophorus nodulosus* occurred with high prevalence in fish sampled from the contaminated sites while these were not found in fish sampled from the reference sites. In particular, the impact of *S. solidus* infection on the selected reproductive parameters was surprisingly higher than expected and this should be taken into account in field monitoring when using the three-spined stickleback as a model species.

The biomarker approach in environmental monitoring is beneficial but must be viewed with respect to evaluation of exposure, effect, and susceptibility. Biomarker strategies must be utilized in an integrated approach in which a hierarchy of responses is evaluated. The analysis of VTG biomarker for estrogens and spiggin biomarker for androgens, together with histopathological analysis in this study reveals evidence that sticklebacks were adversely affected by endocrine disrupters emanating from the sewage treatment plant discharges. The results suggest that fish sampled from most of the localities close

to sewage treatment plants along the Finnish coast of the Baltic Sea receive estrogenic loads sufficient to cause inappropriate production of VTG and to disrupt normal testicular structure in adult male sticklebacks.

5.2. Detection of estrogenic and androgenic effects of municipal wastewater effluent *in vivo* (II)

In a controlled laboratory *in vivo* exposure study with an estrogen (EE2) control, androgen (MT) control, and treated wastewater effluents, the same easily measured biomarkers present in adult three-spined sticklebacks used in the field study were also used to provide a comprehensive set of endpoints for detection of (anti-) estrogenic or (anti-) androgenic activity in municipal wastewater. The studied wastewater treatment plant was treating both domestic and industrial effluent from urban regions in and around the city of Turku, Finland. The aim of this study was to simultaneously assess both the estrogenic and/or androgenic effects of municipal wastewater treatment plant effluents since domestic wastewater effluent is suspected to contain estrogenic and androgenic compounds. The actual concentration of the positive controls EE2 and MT in the aquaria in this study was about 50% of the nominal concentration. The difference from nominal concentration can be attributed to uptake in the fish, microbial activity, photo degradation and/or adhesion to the aquarium and the aquaria system, but also losses during extraction.

5.2.1. Morphometric characteristics

The same morphometric characteristics were measured as in the field study (Suppl. Table 1, II). The time of one and four weeks of exposure did not cause any significant changes in the different morphological parameters measured (CF, GSI, HSI) in any of the treatment groups. NSI was the only index concluded to be a suitable endpoint for detection of androgenic activity in sticklebacks, as the current study showed highly elevated NSI in both female and male sticklebacks exposed to MT.

5.2.2. Biomarkers vitellogenin, spiggin and plasma steroid levels

An induction of VTG production was noted in adult male sticklebacks in response to EE2 exposure at nominal concentration of 20 ng/l (Fig. 1, II). However, the VTG induction was not as significant as described for other species and the sensitivity of VTG inductions seems to vary between different species. Rainbow trout has been found to respond to levels of EE2 as low as nominal concentrations of 2 ng/l (Jobling et al., 1996), adult zebrafish at nominal concentrations of 1.6 ng/l (Fenske et al., 2001), and fathead minnow at nominal concentrations of 5 ng/l EE2 (Panter et al., 2002). The results show that the adult three-spined stickleback is not as sensitive to estrogens as compared to other fish species *in vivo*. However, exposing three-spined sticklebacks in the current

study to wastewater effluent caused an increase in VTG levels compared to control fish, and this effect corresponded to the effect seen in the sticklebacks exposed to EE2, which indicates the presence of estrogenic compounds in the treated wastewater effluent.

The spiggin levels in both male and female adult sticklebacks exposed to MT (nominal concentration 10 μg/l) were significantly elevated (Fig. 2, II). Spiggin levels increased four to five-fold in males after exposure with MT compared to control male fish. For female fish the increase was even more significant since after one week of exposure, the spiggin level was increased over seven-fold compared to the control group. After four weeks of exposure the, spiggin levels were dramatically increased 11-fold. Spiggin induction, especially in females, is therefore concluded to be a good biomarker for androgenic exposure. MT is suggested to be the most potent androgen to induce spiggin in sticklebacks (Katsiadaki et al., 2007), and serves therefore as a good positive control. Wastewater effluent did not cause any significant spiggin induction indicating no clear androgenic activity in the treated wastewater effluent.

No major changes in steroid levels of wastewater effluent exposed fish compared to the control fish were observed (Fig. 3, II), except for elevated T concentrations in both females and males exposed to MT. The reason why no changes in steroid levels in plasma were observed can be due to the effects on steroid biosynthesis which are too minor to be translated into changes in plasma titers as suggested by Sharpe et al. (2004). Alternatively, the exposure time was too short to cause any significant changes in steroid synthesis.

5.2.3. Histopathology

Female fish exposed to EE2 and wastewater effluent showed an increased number of atretic follicles in the ovaries and a decreased number of vitellogenic follicles relative to control fish (Fig. 4, II). Similar effects have also previously been observed in zebrafish exposed to estrogens (van den Belt et al., 2002; van der Ven et al., 2003). After exposure to MT, mature eggs accumulated in the ovary and MT appeared to have an inhibitory effect on ovulation. This has also been observed in zebrafish exposed to 10 μg/l 17α-methyl-dihydrotestosterone (van der Ven et al., 2003). The histological examination of testes revealed severe effects on the testicular structure in response to exposure to EE2 and wastewater effluent (Fig. 5, II). Testes of exposed fish were also affected by the low (10%) and medium (50%) concentration of wastewater effluent. Spermatogenesis was impaired and the seminiferous lobules of the testis were degenerated. There was also increased interstitial fibrosis observed, which previously has been seen after estrogenic exposure of adult male fish (Gray et al., 1999; Karels et al., 2003; Rasmussen et al., 2005). Macrophage aggregates appearing as yellowish-brown pigmented structures were also present in testis from the wastewater treatment groups. The treatments of sticklebacks with MT did not cause any obvious imbalance in the spermatogonic classes in this

study. The masculinising effect of MT was also reflected in the VTG response since the VTG levels were decreased in females exposed to MT in comparison to the control fish. This study confirms histological changes in fish associated with exposure to hormones and wastewater effluent. Histological changes were seen already with low or medium concentration of wastewater effluent. This is in contrast to the VTG response in which no significant effect was observed in the fish exposed to low or medium concentrations of wastewater effluent. Thus the threshold for VTG induction in stickleback by wastewater effluent was higher than that for induction of testicular changes.

In conclusion, the current study indicated that municipal wastewater effluent, in higher concentrations, exerted estrogenic action in adult stickleback. This is based on the results showing elevated VTG levels, and histopathological effects on testis corresponding to the effects seen in the fish exposed to EE2.

5.3. Comparative studies on exposures in the field and controlled *in vivo* exposures (I&II)

Local environmental differences favouring earlier spawning or more abundant food supply can cause differences in the condition indices. Interestingly, the somatic data including condition indices appeared to be very similar for the field collected fish and the laboratory exposed fish. It is worth noting that field collected female fish had significantly higher GSI compared to the laboratory exposed females. However, GSI is not comparable in this case since most of the females in the laboratory exposure had ovulated during the exposure. The potential androgen biomarker NSI was shown to be very efficient in detecting androgen (MT) exposure in the laboratory study. However, similar responses in NSI in field collected fish were not observed, which suggests no presence of androgen compounds in the field.

To place data in the context of the laboratory based exposure study, VTG was measured in blood plasma of sticklebacks exposed to 20 ng/l EE2 (nominal concentration) to be 6.8±1.2 µg/ml. For male sticklebacks exposed to wastewater effluent in the laboratory, the mean measured VTG concentration ranged between 1.4 and 5.6 µg/ml. These levels relate to baseline concentrations in control male sticklebacks of 1.3±0.2 µg/ml (study II). Male sticklebacks caught from the uncontaminated sites in the field had VTG levels around 1.3 µg/ml, corresponding to control fish in the laboratory exposure study. Sticklebacks caught from the contaminated sites had values ranging from 3.6-6.3 µg/ml, corresponding to wastewater effluent treated fish in the laboratory exposure study.

When comparing spiggin levels from males in the field study and the laboratory experiment it became evident that the baseline concentrations were higher in the field captured fish (in average 150 A.u/mg) compared to laboratory exposed control fish

(50 A.u/mg). This is likely due to the lack of nest building material and subsequent courting behaviour in the controlled aquaria exposure study, leading to decreased spiggin levels. However, male fish exposed to MT (nominal concentration 10 µg/ml) showed significantly increased levels of spiggin (250 A.u/mg). Female fish exposed to MT showed dramatically increased spiggin levels up to 500 A.u/mg compared to control females having 30 A.u/mg. Such levels were never observed in the field study suggesting that there is no androgen exposure present in the studied environments.

The histological assessment of gonads from field collected fish and laboratory exposed fish revealed high similarity in the changes seen in testis (Table 1). All male fish exposed in laboratory conditions to EE2, different concentrations of wastewater effluent (Fig. 5, II), or male fish caught from the contaminated sites in the field study (Fig 3, I) showed similar changes, such as impaired spermatogenesis and degenerated lobules of the testis. It can therefore be concluded that the observed changes can be explained by estrogenic compounds present in the water.

Table 1. Histopathological categorization of testis analysed in the field study and in the laboratory exposure study. The sites used in the field study are; A Molpe (reference site), B Nagu (reference site), C Jakobstad (STP), D Vasa (STP), E Dalsbruk (STP), F Mariehamn (STP). STP indicates that the sampling was done next to a sewage treatment plant. The treatments in the laboratory exposure study are at the start of the exposure experiment (0-sample), after exposure to control water (Control), 20 ng/l 17α-ethynylestradiol (EE2), 10 µg/l 17α-methyltestosterone (MT) and different concentrations (10, 50 and 80%) of wastewater effluent for a time period of four weeks.

Treatment	Presence of phagocytising Sertoli cells	Disruption of lobular arrangement	Interstitial fibrosis	Impaired spermato-genesis	Summary of pathological changes
Site A (ref)	+	–	–	–	–
Site B (ref)	–	–	–	–	–
Site C (STP)	+	+	+	+	+
Site D (STP)	++; PC	++	++	++	++
Site E (STP)	+	–	+	–	+
Site F (STP)	+++; PC	+++	+++	+++	+++
0-sample	–	–	–	–	–
Control	+	–	–	–	–
20 ng/l EE2	+++	+++	+++	+++	+++
10 µg/l MT	–	–	–	–	–
10% sewage	++	–	++	+	+
50% sewage	++; PC	+	++	++	++
80% sewage	+++	+++	+++	+++	+++

Abbreviation used: – normal; +, ++, +++ indicates increasing effect; PC - yellowish-brown pigmented cells.

The results show that several parameters need to be combined for detection of potential endocrine modulating substances using the three-spined stickleback as models organism. Even though histopathology has often been considered a tedious method, it proved to be most sensitive parameter together with VTG and spiggin analysis. Parasite infections

are also important to consider since infections impose energetic costs of the host and can increase mortality. Parasite infections can be avoided by use of laboratory bred sticklebacks for *in vivo* exposure studies. In summary, the results indicate that endocrine disruption in receiving waters of sewage effluents in coastal areas of Finland may not be such a widespread problem as described in other countries (reviewed in Jobling and Tyler, 2003). Compared to many other regions in Europe, Finland is relatively sparsely populated which reflects in lower input volumes to the sewage treatment plants. The sewage treatment plants in Finland have in general high technical standards with multiple treatment steps. The dilution factors in receiving waters also reduce the exposure of aquatic organisms. However, estrogenic effects were observed, which points out that dilution is not the solution to pollution. No clear androgenic disruption was observed in sticklebacks.

5.4. Development of estrogen and androgen sensitive bioassays *in vitro* (III)

The aim of the study was to develop rapid *in vitro* screening techniques in order to simultaneously assess estrogenic and androgenic activity of chemicals and environmental samples. The bioassays were developed using cell and tissue preparations from three-spined stickleback. Cells or tissues isolated from a number of different fish species have previously been evaluated for the determination of endocrine disruption *in vitro.* These cells or tissues have mainly been used for the valuation of estrogenic effects (Iguchi et al., 2006). Despite increasing concern about effects due to anthropogenic compounds having androgenic or anti-androgenic properties, examination of androgenic effects using fish cells has been insufficiently assessed. Therefore, high priority was directed towards the development of *in vitro* assays for environmental androgens.

The measured endpoints in this study included the production of VTG as a marker for estrogen potency and the production of spiggin as a marker for androgen potency of the compounds tested. The developed primary cell culture tests can be carried out within five days and the tissue slice culture test in two days, compared to a standard *in vivo* test that normally needs a minimum of 21 days of exposure. The tests are based on microtiter plate assays allowing high number of chemicals or environmental samples to be examined simultaneously. *In vitro* assays with primary cell- and tissue slice cultures offer a more rapid response and lower equipment requirements compared to conventional *in vivo* exposure procedures using whole animals.

5.4.1. Estrogen sensitive bioassay

The most useful *in vitro* system for studying environmental estrogens are fish primary hepatocyte cultures with the induction of VTG by E2 being the most studied endpoint

(Bols et al., 2005). Cytological characterizations have shown that fish hepatocytes in primary culture display only minor cytological alterations during the first five days (Pesonen and Andersson, 1997). Primary cultured fish hepatocytes have been used in selected OECD fish species, but the use of cultured hepatocytes from stickleback has not been reported earlier. In study III, stickleback hepatocytes responded very well when treated *in vitro* with E2, with the maximal peak induction at 400 nM (Fig 2, III). The dose-response relationships for VTG induction by E2 were similar for hepatocytes of males and females, even though the absolute quantities of VTG produced by the treated cells of female origin were higher than in similarly treated cells from males. EE2 was shown to be more potent than E2 in inducing VTG production, which has previously been observed in other fish hepatocyte cultures (Pelissero et al., 1993; Kordes et al., 2002). Male liver tissue slice cultures responded positively with induced VTG production after exposure for 10 μM E2 (Fig 4A, III). VTG production was not obtained with E2-treated female liver tissue slice cultures with the tested concentrations. Based on the sensitivity level, the hepatocyte cultures should be used in favour of tissue slice cultures for the evaluation of estrogen activity. The responsiveness of male hepatocyte cell cultures to estrogens lead to a higher magnitude of VTG induction compared to female hepatocyte cells. Male hepatocyte cell cultures are therefore considered to be more suitable in detecting estrogenic activity. The primary cell and tissue slice cultures prepared from stickleback were also tested for their capability to detect activities in environmental samples, and the same outgoing sewage water from the city of Turku used in the *in vivo* exposure study (II) was analyzed. With hepatocyte cell cultures, derived from both male and female three-spined sticklebacks, a dose-dependent response of estrogenic activities was detected in the sewage water comparable to the maximum obtained E2 induced response (Fig. 5A, III).

5.4.2. Androgen sensitive bioassay

The use of stickleback kidney cell cultures with the induction of the androgen-dependent protein spiggin for evaluation of androgenic activity is a new approach for investigating androgen effects of hormones and environmental samples. There are several cell types present in the stickleback kidney but it is presumably the tubule epithelial cells that produce spiggin. Spggin producing cells were identified with immunohistochemistry, and 70-80% of the kidney cells were shown to be immunoreactive in the kidney cell cultures. Male kidney cells responded positively to *in vitro* treatment of androgens, with the synthetic androgen MT being more potent than the natural androgen 11-KT (Fig. 3A, III). Female kidney cells also responded to androgen treatment in the same pattern but demanded higher concentrations of androgens (Fig. 3B, III). This may be due to the fact that the male kidney cells were isolated from mature male fish and were already in a hypertrophied state while female cells were only photoperiodically activated and not androgen-primed. Priming with hormones might have induced higher spiggin levels in

kidney cell cultures, which previously were demonstrated for three-spined stickleback females exposed for DHT during ten days to stimulate kidney hypertrophy prior to cell preparations (Jolly et al., 2006). On the other hand, kidney tissue slice cultures responded to androgen treatment very rapidly, with lower concentrations of androgens used and with a significant higher magnitude of response compared to kidney cell cultures (Fig. 4B, III). The advantage of tissue slice culture over isolated kidney cells is that the tissue architecture is preserved and the tissue pieces contain all cell types being present in the intact kidney. Unprimed female kidney tissue slice cultures in favour of cell cultures could therefore be a suitable assay to test androgenic effects due to the high spiggin response obtained when exposed to 11-KT and the rapid assay time of only two days. No androgenic activity was however detected in sewage water when tested on stickleback kidney cells or tissue slice cultures (Fig. 6, III), which indicates that no androgen compounds were present in the tested wastewater effluent.

The results of study III confirm the estrogenicity of the municipal wastewater effluent previously observed in study II, but the present assay does not give an indication on the identity of the active compounds in the wastewater causing the effect.

5.5. Identification of estrogenic activity in municipal wastewater (IV)

The steroid estrogens E2 and E1 and occasionally EE2 has emerged as the most important estrogenic compounds in effluent, with concentrations between 2.7-48 ng/l E2, 1.4-76 ng/l E1 and up to 7 ng/l EE2 (Johnson et al., 2005). The estrogenic activity of steroid estrogens has also been shown in a variety of *in vivo* studies conducted in fish (Routledge et al., 1998; Länge et al., 2001; Jobling et al., 2002 Thorpe et al., 2003). To identify individual estrogenic compounds in environmental samples, the results from biological assays must be combined with chemical analysis (gas or liquid chromatography coupled to mass spectrometry, GC-MS, LC-MS). In study IV, a solid phase extraction and a chromatographic fractionation of the wastewater effluent was performed prior to assessment of estrogenic activity using VTG induction by primary hepatocyte cultures prepared from male three-spined stickleback. These results in combination with LC-MS/MS analysis were used for the determination of the compounds accounting for the estrogenic activity observed previously (study II, III) in the wastewater effluent.

The lowest effective concentration (LOEC) for stickleback hepatocytes was shown to be 1 nM E2 (Fig. 3, IV). The LOECs for VTG induction in cultured rainbow trout and common carp hepatocytes have been shown to be 0.1 nM (Jobling and Sumpter, 1993) and 2 nM E2 respectively (Smeets et al., 1999), suggesting that stickleback male hepatocytes are in the same range of sensitivity. Chemical analysis of the wastewater effluent showed that the concentrations of E1 were 65 ng/l and E2 and EE2 were at or under the limit of quantification, i.e. less than 0.7 ng/l and 1 ng/l, respectively (Table 1,

IV). E2 values in the effluent were lower than E1 values. This trend would be expected, since E2 is less excreted by humans (Johnson et al., 2000) and E2 appears to be more rapidly degraded than E1. E2 degradation will also generate E1 as a by-product. The generally low concentration of E2 in effluents is also reported in other surveys (Belfroid et al., 1999; Baronti et al., 2000; Spengler et al., 2001; Johnson et al., 2005). The method used was perhaps not sensitive enough to detect EE2 but because its stability in the environment and potency *in vivo* and *in vitro* it is nevertheless a relevant endocrine disrupter in effluents. However, when solid phase extracts of the wastewater and HPLC fractions of the extract were assayed with the male hepatocyte assay, it was shown that it is not only the estrogens E1, E2 and EE2 that account for the estrogenic activity in the wastewater effluents (Fig. 4, IV). Only a minor contribution to the activity could be derived from these compounds. These findings are contradictory to the results of other studies where it is found that the natural estrogens are the major contributors to the estrogenicity of wastewater effluents and of surface water (Desbrow et al., 1998; Ternes et al., 1999; Snyder et al., 2001; Aerni et al., 2004; Furuichi et al., 2004; Nakada et al., 2004; Beck et al., 2006; Roda et al., 2006). This work implies that the examined wastewater effluents contain compounds with estrogenic activity, other than the steroidal estrogens measured. The observed activity might be due to synergistic or additive effects of the steroidal estrogens measured and/or of unknown compounds present in the effluent. It has previously been shown that besides the natural estrogens and the synthetic steroidal estrogens, non-steroidal synthetic compounds like nonylphenol and phthalates, and animal sterols (i.e. cholesterol and its derivatives) occur at measurable levels in wastewater effluents (Fernandez et al., 2007).

5.6. Comparative studies on *in vivo* and *in vitro* exposures

In vitro assays can provide valuable insights on mechanisms of action but their capacity to mimic whole animal uptake, metabolism, distribution and targeting of (xeno) estrogens and (xeno)androgens is restricted. Therefore, caution needs to be taken when interpreting *in vitro* results without *in vivo* confirmation, since extrapolation from *in vitro* to *in vivo* systems may lead to false negatives or overestimation of the potency of compounds. The most significant findings in the present studies were changes in plasma VTG concentrations, spiggin concentrations, changes in the NSI and developmental stages of sperm cells in testis of stickleback exposed to municipal wastewater effluent or pure hormones. Of these parameters, changes in VTG synthesis and spiggin synthesis were possible to be detected with the *in vitro* assays. The primary hepatocytes were able to predict the estrogenicity of pure estrogens but also municipal wastewater effluent through elevated VTG levels. The *in vitro* test was detecting significant estrogenicity of a medium (50%) and a high concentration (80%) of effluent, corresponding to the effects seen *in vivo* with the histopathological changes, while VTG levels *in vivo* exposed fish

showed elevated levels of VTG only when exposed to the high concentration (80%). The kidney tissue slice cultures were able to detect androgen action of pure hormones in the same manner as in the *in vivo* exposure study. No androgenicity was detected in municipal wastewater effluent either *in vivo* or *in vitro*. In contrast to the estrogen receptor that is mostly activated by compounds present in environmental samples, the androgen receptor seems to be prone to antagonism rather than agonism (Sonneveld et al., 2005), therefore anti-androgenic properties of EDCs should be further investigated. It can therefore be concluded that the stickleback hepatocyte VTG *in vitro* assay could predict the responses of municipal wastewater effluent in fish *in vivo*, and the stickleback spiggin *in vitro* assay should be developed further as sensitive indicator assay for detection of especially anti-androgenic action of endocrine modulating substances.

6. CONCLUDING REMARKS

Considerable homology exists in the endocrinology of vertebrates; hence, toxicants that alter endocrine function in one species are likely to produce adverse effects in another. However, there are significant differences between some species in endocrine functions that calls out consideration for further interspecies extrapolation. It is important to remember that although the hormones, hormone synthesis and their receptors are highly conserved, the role of specific hormones in reproductive function and development can vary greatly.

This thesis shows that compounds with estrogen activity can be found in effluent of domestic sewage treatment plants in Finland, and the environmental impact of these compounds will depend on their dilution in the receiving waters in each location. The municipal wastewater effluent affected plasma VTG, spiggin levels and gonad histopathology in the three-spined stickleback, which was used as model species. This thesis emphasizes the need for a multi-parameter approach in detecting endocrine disrupting properties *in vivo* in fish, where the rapidly assessed biomarkers such as VTG and spiggin should be evaluated together with more complex biomarkers such as gonad histopathology. It is also important that we adopt a broader perspective and consider the biological context in which exposure occurs e.g. mechanism of action, timing of exposure, life exposure, susceptible life stages, dose, duration, and mixture effects when assessing exposure risk.

The European Commission's proposed Registration, Evaluation, and Authorisation of Chemicals (REACH) programme is a comprehensive, precautionary-based approach to regulate chemicals. The chemical regulation will improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances. However, this may lead to an increase in animal use since there are today over 100,000 existing substances that have been used without any testing. Animal testing of these chemicals would be extremely costly and time demanding. Sensitive *in vitro* methods for screening chemicals including endocrine disrupting activities is therefore very much needed. The strong need for alternative methods in assessing effects of endocrine disrupting chemicals was explored in this thesis through the development of specific *in vitro* screening assays for detection of estrogenic and androgenic effects. It was shown that the *in vitro* assays were able to predict the effects of pure hormones and complex environmental samples such as municipal wastewater effluent with reasonable accuracy. The *in vitro* tests could therefore be used as early screening tools before *in vivo* confirmation.

Wastewater effluent was shown to exert estrogenic effects on adult stickleback both *in vivo* and *in vitro*. When the effluent was analysed it was shown to contain compounds

with estrogenic activity, other than the steroidal estrogens measured. The observed activity could have been due to synergistic or additive effects of the steroidal estrogens measured and/or of unknown compounds present in the effluent. It is therefore important to recall that estrogenic activity in wastewater effluent could be underestimated when the activity of one or a few compounds are considered or if theoretical estrogenic activity is the sum of the activity of each single compound.

This thesis indicates that endocrine disruption in coastal areas receiving sewage effluents in Finland using the stickleback as model species may not be a widespread problem. A combination of relatively low endocrine activity in the effluents, controlled discharges of wastewater and a high dilution rate in the receiving waters may explain these results. However, endocrine disruption might still be of local concern in recipients with low dilution rates.

There are very few fish species that can match all the advantages that the stickleback presents as a model species in endocrine disruption research. Therefore, this model species will hopefully contribute significantly to the assessment of the impact of endocrine disruptors not only on wildlife but also on human health.

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