The Zebrafish as a Model Organism for Evaluation of Endocrine Disrupters

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Abstract

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Many of the natural, pharmaceutical and industrial chemicals that are present in the aquatic environment can interfere with the endocrine system of fish. There is a demand for sensitive and robust test systems for risk assessment of the endocrine disrupting chemicals (EDC). The aim of this thesis was to develop a partial life-cycle test to be used for evaluation of EDC. The test was based on exposure of juvenile zebrafish to EDC during the period of gonad transformation and differentiation. The main endpoints studied were the induction of the estrogenic biomarker protein vitellogenin (Vtg) and gonad development.

Zebrafish were exposed to single model substances to investigate if exposure during the period of gonad transformation and differentiation affects Vtg production and gonad development. Exposures to the synthetic estrogen 17α-ethinylestradiol (EE2) resulted in a dose-dependent increase in Vtg production, as well as female-biased sex ratios, including all-female stocks. Exposures to the androgens 17α-methyltestosterone (MT) and 17β-trenbolone (Tb) resulted in decreased Vtg concentrations, as well as male-biased sex ratios, including all-male stocks. In a comparative study with Japanese medaka a higher sensitivity of both Vtg and sex ratio of the zebrafish was shown after exposure to EE2 and Tb. Exposures to binary mixtures of the differently acting substances EE2 and Tb demonstrated that effects on both Vtg and gonad development can be detected. Application of this zebrafish partial life-cycle test on a chemically complex pulp mill effluent revealed both estrogenic and androgenic effects. In conclusion, the zebrafish respond to EDC at environmentally realistic concentrations. The results support that zebrafish is a suitable model species for evaluation of EDC.

Keywords: zebrafish, *Danio rerio*, endocrine disrupters, gonad development, sex ratio, vitellogenin

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Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. S. Örn, H. Holbech, T.H. Madsen, L. Norrgren, and G.I. Petersen (2003) Gonad development and vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and methyltestosterone. Aquatic Toxicology 65:397-411

II. S. Örn, S. Yamani, and L. Norrgren (2006) Comparison of vitellogenin induction, sex ratio and gonad morphology between zebrafish and Japanese medaka after exposure to 17α-ethinylestradiol and 17β-trenbolone. Archives of Environmental Contamination and Toxicology, accepted for publication

III. S. Örn, H. Holbech, and L. Norrgren (0000) Effects on vitellogenin production and gonad development in zebrafish (*Danio rerio*) exposed to mixtures of 17β-trenbolone and 17α-ethinylestradiol. Manuscript

IV. S. Örn, A. Svenson, T. Viktor, H. Holbech, and L. Norrgren (2006) Male-biased sex ratios and vitellogenin induction in zebrafish (*Danio rerio*) exposed to pulp mill effluent. Archives of Environmental Contamination and Toxicology, accepted for publication

Abbreviations

ANOVA	analysis of variance
DHT	dihydrotestosterone
E1	estrone
E2	17β-estradiol
EDC	endocrine disrupting chemicals
EE2	17α-ethinylestradiol
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
HE	haematoxylin-eosin
LM	light microscopy
MDHT	17α-methyldihydrotestosterone
MeOH	methanol
MT	17α-methyltestosterone
PME	pulp mill effluent
STW	sewage treatment work
Tb	17β-trenbolone
TEM	transmission electron microscopy
Vtg	vitellogenin
Wph	weeks post-hatch

Introduction

General introduction

Many chemicals produced by human activities end up in the aquatic environment. Aquatic animals living in polluted areas, such as fish, might be continuously exposed throughout their life to a multitude of different chemicals originating from industrial, agricultural and domestic sources. During the last decade the role of substances that can interfere with the endocrine system of an individual has been in focus, the endocrine disrupting chemicals (EDC). EDC exert their effects by mimicking or antagonizing endogenous hormones, altering the natural pattern of hormone synthesis or metabolism, or modifying hormone receptor levels (Sonnenschein & Soto 1998). A variety of anthropogenic chemicals have been shown to act as EDC, including high volume products such as phthalates, bisphenol A, and alkylphenols (Sonnenschein & Soto 1998, Tyler *et al*. 1998, Vos *et al*. 2000). Moreover, among pharmaceuticals and phytosteroids many substances mimick the endogenous hormones (Sonnenschein & Soto 1998, Tyler *et al*. 1998, Vos *et al*. 2000). In addition to the risk of exposure to single substances, complex mixtures present in domestic and industrial effluents must be considered.

Environmental estrogens

Most studies on EDC have focused on effects of chemicals acting through the same mechanisms as endogenous estrogens. Natural endogenous estrogens, such as estrone (E1) and 17β-estradiol (E2), as well as the synthetic estrogen 17α-ethinylestradiol (EE2) have frequently been detected in effluents from domestic sewage treatment works (STW) in Europe, North America and Australia (Desbrow *et al*. 1998, Belfroid *et al*. 1999, Ternes *et al*. 1999, Braga *et al*. 2005, Johnson *et al*. 2005, Servos *et al*. 2005). Concentrations of estrogens in effluents are often detected in the lower ng/L range. For instance, a survey of Italian STW found median effluent concentrations of E2 at 1 ng/L and EE2 at 0.45 ng/L (Baronti *et al*. 2000). In effluent water from a Swedish STW, concentrations of E1, E2, and EE2 were 5.8, 1.1, and 4.5 ng/L, respectively (Larsson *et al*. 1999). However, occasionally, much higher effluent concentrations of estrogens have been measured, such as 220 ng/L of E1, 88 ng/L of E2 and 42 ng/L of EE2 (Desbrow *et al*. 1998, Ternes *et al*. 1999). In a nationwide survey of 139 streams in USA, the maximum and median levels for E1 were 112 and 27 ng/L, and for E2 200 and 160 ng/L (Kolpin *et al*. 2002). Maximum concentrations of EE2 as high as 831 ng/L were measured, with median concentrations of 73 ng/L (Kolpin *et al*. 2002).

Adverse effects of EDC on wild fish are usually unknown although some links have been shown, especially to xenoestrogens. During the 1990s, the observation of intersex fish in UK rivers initiated a number of studies to determine the cause. Several studies showed that in fish placed in cages, as well as wild fish, downstream of STW the levels of the estrogenic biomarker protein vitellogenin (Vtg) were elevated (Purdom *et al*. 1994, Harries *et al*. 1996, Harries *et al*. 1997,

Lye *et al*. 1997). Further studies showed widespread occurrences of intersex in wild roach (*Rutilus rutilus*) populations, which was more pronounced at the outlets of STW (Jobling *et al*. 1998) linking the occurrence of intersex with the presence of estrogens in the sewage effluent. Later studies of laboratory exposures of roach to estrogenic STW effluents during sex differentiation resulted in increased vitellogenin concentrations as well as feminisation of the reproductive tract (Rodgers-Gray *et al*. 2001, Liney *et al*. 2005). Since the findings in UK, increased vitellogenin production in wild or caged fish on exposure to STW effluents has been reported in a number of studies (e.g. Folmar *et al*. 1996, Larsson *et al*. 1999, Svenson *et al*. 2002). Apart from domestic effluents, increased vitellogenin production has also been shown in connection with exposure to pulp mill (Mellanen *et al*. 1999, Tremblay & Van der Kraak 1999, Van Den Heuvel & Ellis 2002) and oil refinery effluents (Knudsen *et al*. 1997). Intersex in wild roach has also been observed in other European countries, including Finland, Sweden and Denmark (Wiklund *et al*. 1996, Andersen *et al*. 2001, Bjerregaard *et al*. 2005). Like in UK, in Danish streams sewage effluent load was found to positively correlate with roach intersex ratio (Bjerregaard *et al*. 2005). Intersex has also been described in other wild fish species, such as barbel (*Barbus plebejus*), bream (*Abramis brama*), gudgeon (*Gobio gobio*), flounder (*Platichthys flesus*) and carp (*Cyprinus carpio*) (Vigano *et al*. 2001, Vethaak *et al*. 2002, Van Aerle *et al*. 2001, Hashimoto *et al*. 2000, Matthiessen *et al*. 1998, Allen *et al*. 1999, Sole *et al*. 2002). How intersexuality in fish may affect reproduction has not been clarified. However, roach with intersex have been shown to have impaired reproduction capacity, with reduced milt volume, reduced sperm density, and reduced fertility (Jobling *et al*. 2002a, Jobling *et al*. 2002b).

Environmental androgens

Although estrogens have received most attention, androgens have also been detected in the aquatic environment. Studies of *in vitro* androgenic activity, based on the yeast androgen screen assay, in seven different UK estuaries and identification of the responsible substances revealed several natural androgenic steroids and their metabolites (Thomas *et al*. 2002). In one estuary receiving sewage effluent discharge, 4-androstenedione and its metabolite 5α-androstanedione were identified as major contributors to the androgenic activity (Thomas *et al*. 2002). Estimated concentrations of androstenedione and androstanedione in the effluent were 105 and 96 ng/L, respectively (Thomas *et al*. 2002). Moreover, pulp and paper mill effluents in Sweden have been demonstrated to display androgenicity using the same *in vitro* assay (Svenson & Allard 2004). Androstenedione, as well as testosterone have also been detected in effluents from several domestic STW in USA (Kolodziej *et al*. 2003), in pulp mill effluents (Jenkins *et al*. 2001, Durhan *et al*. 2002, Jenkins *et al*. 2003) and in streams receiving runoff from areas with animal production (Kolodziej *et al*. 2004, Shore *et al*. 2004). In a nationwide survey of 139 streams in USA, testosterone was measured at a maximum concentration of 214 ng/L, with a median concentration of 116 ng/L (Kolpin *et al*. 2002). Feedlot effluents with androgenic *in vitro* activity have been connected with the presence of metabolites of trenbolone acetate, which is used as a synthetic androgenic growth promoter in beef cattle

production in North America (Orlando *et al*. 2004, Soto *et al*. 2004, Wilson *et al*. 2002, Lange *et al*. 2001).

Historically, pulp and paper mill effluents have caused various disorders in fish. Despite process changes and waste water treatment improvements during the 1990s, endocrine effects are still documented (Munkittrick *et al*. 1998, Parrot *et al*. 2004, Kovacs *et al*. 2005). A well-known example of masculinisation of wild fish is the mosquitofish (*Gambusia affinis*) living in streams in Florida, USA. In Fenholloway River, which receives paper mill effluents, female mosquitofish have been reported to develop an elongated anal fin resembling the male gonopodium (Howell *et al*. 1980, Cody & Bortone 1997, Bortone & Cody 1999, Jenkins *et al*. 2001). The cause to this masculinisation is still unknown, and the responsible substances have not been identified. The androgen androstenedione has been detected both in the water and sediment of Fenholloway River (Jenkins *et al*. 2001, Jenkins *et al*. 2003). However, androstenedione seems not to be the major contributor to the androgenic activity, and other non-identified androgens are known to be present (Parks *et al*. 2001, Durhan *et al*. 2002). Masculinisations of fish have also been observed on exposure to effluent water from a Swedish pulp mill. In 1998, Larsson *et al*. (2000) observed male-biased sex ratios in the offspring of eelpout (*Zoarces viviparus*) sampled in the vicinity of the pulp mill. In a further study on historical samples of eelpout, they observed male-biased sex ratios at the same location in 1997, 1998 and 2000 (Larsson & Förlin 2002). In 1999, normalised sex ratios were observed. This recovery was related to a 17-day shutdown at the mill, coinciding with the period of gonadal differentiation in eelpout embryos (Larsson & Förlin 2002). In laboratory experiments, female guppies exposed to a 10% dilution of the effluent displayed enhanced coloration, indicating an androgenic response (Larsson *et al*. 2002). The androgenicity has also been confirmed using female three-spined sticklebacks (*Gasterosteus aculeatus*). Increased epithelial cell height and increased production of the glue protein spiggin in the kidneys are androgen regulated processes normally occurring in male sticklebacks during reproduction. These androgenic effects were detected in females after exposure to 10% pulp mill effluent for six weeks (Katsiadaki *et al*. 2002).

Fish as models for evaluating EDC

Single estrogens and androgens have generally been detected in aquatic environments at concentrations below the effect levels reported from laboratory studies. However, EDC are commonly present in combination with each other and with many other differently acting substances. The effects on fish of combinations of EDC have not been well studied. A few mixture studies in the literature focus on estrogenic chemicals using vitellogenin production as the endpoint (e.g Brian *et al*. 2005, Thorpe *et al*. 2001, Thorpe *et al*. 2003). However, combinatory studies with dissimilarly acting EDC are limited, and effects other than Vtg production have not been well studied.

Extrapolation between laboratory fish studies and observations on effects in wild fish is important for evaluation of individual chemicals and complex mixture of chemicals, such as sewage effluents. The zebrafish is together with the Japanese

medaka (*Oryzias latipes*) and the fathead minnow (*Pimephales promelas*) considered as model test species for risk assessment of EDC (Ankley & Johnson 2004, OECD 2004). Endpoints that are used to evaluate EDC include vitellogenin, gonad differentiation, sex ratios, and reproduction success.

The zebrafish (*Danio rerio*; former *Brachydanio rerio*) is a tropical cyprinidae species native to rivers in India and Pakistan (Laale 1977). The species has been used as a vertebrae model predominantly in developmental biology, genetics and toxicology. In molecular genetics the sequence of the genome is nearly completed (www.sanger.ac.uk) and several hundred of zebrafish mutant strains are commercially available. The small size (4-5cm) of zebrafish benefits reductions in holding space and husbandry costs, as well as minimisations in the use of chemicals. Moreover, the small size promotes whole-body sectioning with histological evaluation of all organs in the body. The high year-round fecundity, with one female capable of laying 200-300 eggs/day, and the short time to reach sexual maturation (3-4 months) makes generational studies with large number of individuals possible. Furthermore, the optical transparency of non-adherent eggs, together with a rapid early development, i.e. 3-4 days from fertilisation to hatch, makes the zebrafish suitable for embryo-toxicological studies.

Sex determination and gonad differentiation in fish

In fish, sex determination on the genetic basis is poorly understood. Both male (XX/XY) and female heterogamety (ZZ/ZW), with the W chromosome carrying an ovary-determining factor, exist. Moreover, multiple sex chromosomes, polygenic sex determination, and environmental sex determination are known (Devlin & Nagahama 2002). Similar to mammals, the Japanese medaka has XX/XY sex chromosomes. The male sex-determining gene has been identified in medaka, and was suggested as the analogue of the mammalian gene (Matsuda *et al*. 2002, Nanda *et al*. 2002). However, in some strains of medaka males are missing the gene, which suggests that other factors might contribute in the sex determination (Nanda *et al*. 2003). In zebrafish the mechanism of sex determination is unknown. The zebrafish has been reported to lack sex chromosomes, and environmental or polygenic sex determination has been proposed (Amores & Postlethwait 1999, Traut & Winking 2001, Wallace & Wallace 2003). In contrast, a study on wild zebrafish from Mansar Lake, India, revealed the presence of sex chromosomes and that the species display female heterogamety (Sharma *et al*. 1998). In gynogenesis studies, using eggs fertilised with sperms whose genetic material has been inactivated, male zebrafish can still be produced, with sex ratios even strongly male biased, supporting the notion that zebrafish lack sex chromosomes (Pelegri & Schulte-Merker 1999). Temperature has been suggested as a factor affecting sex ratios in zebrafish. Uchida *et al*. (2004) reported that at temperatures of 35°C and 37°C more males were produced. Wallace & Wallace (2003) reported no evidence for temperature-dependent sex determination when rearing zebrafish at temperatures between 20°C and 32°C.

In fish, sexual differentiation is highly variable (reviewed by Devlin & Nagahama 2002). Many species are gonochorists, where individuals develop as males or

females, and remain the same sex throughout their life. In differentiated gonochoristic species, also termed primary gonochorism, early gonad development proceeds from an undifferentiated gonad directly to ovary or testis. Secondary gonochoristic fish species experience a period where all gonads are initially intersexual prior to differentiation into either testis or ovary. In undifferentiated gonochoristic species, such as the zebrafish, all individuals initially develop ovarian tissue. In contrast to gonochorists, hermaphrodite fish can produce both male and female gametes sometime in their lives. In synchronous hermaphrodites male and female gametes are produced at the same time, and in sequential hermaphrodites individuals first produce one gamete type, then reverse sex and produce the other type of gamete (Devlin & Nagahama 2002). Zebrafish are considered to belong to undifferentiated gonochoristic species. All individuals first develop undifferentiated ovaries. In individuals becoming males the oocytes degenerate, and the gonad then develops into a phenotypic testis. This phenomenon was termed juvenile hermaphroditism when first described by Takahashi (1977). However, differences in the period of gonad transformation from ovaries into testes have been observed. The disappearance of oocytes in the transforming gonad has been reported to take place between 3-4 weeks post-hatch (Takahashi 1977, Uchida *et al*. 2002). Other studies have reported this transformation period to be more extended (Maack & Segner 2003, Brion *et al.* 2004). Once the transformation is completed, the gonads develop into phenotypic ovaries or testis, and sexual maturation is reached at approximately 2-3 months.

Fish gonads are structurally similar as other vertebrates, with germ cells intermixed with supporting somatic cells. During development of the ovary, the somatic cells and germ cells differentiate to form follicles; oocytes surrounded by an inner granulosa and outer theca layer. In the theca layer, testosterone and other precursor androgens are produced. In the granoulosa layer aromatisation of testosterone into estrogen takes place, with 17β-estradiol being the main female estrogen. In zebrafish, the development of the oocytes has been divided into five different stages from the primary growth stage (Stage I) to mature eggs (Stage V) which are ovulated into the ovarian lumen and ready for fertilisation (Selman *et al*. 1993). The ovary of zebrafish is bilobulated and belongs to the cystovarian type, having a true ovarian cavity. The eggs are ovulated into the ovarian lumen, which is continuous with a short oviduct that leads to the genital opening posterior to the anus (Selman *et al*. 1993). In the testis, the somatic cells differentiate into seminiferous tubules and supporting connective tissue, and to Leydig and Sertoli cells. The Leydig cells are the main site of androgen synthesis. In fish, testosterone and especially 11-ketotestosterone are the main androgens (Borg 1994). Sertoli cells support the development of the spermatozoa and secrete androgen binding proteins. The structure of the testis in zebrafish has been described by Ewing (1972). The paired testes are located along the lateral body wall. Approximately 5 parallel efferent ducts run dorsally the length of each testis collecting spermatozoa from the seminiferous tubules. The efferent ducts unite at the genital papillae. The seminiferous tubules are of the anastomising type, with spermatogonia distributed along the entire length of the tubules. Seminiferous cysts, with synchronously developing germ cells, break when the cyst is mature and release spermatozoa into the tubule lumen.

Effects of exposure to EDC in zebrafish

Estradiol stimulates oocyte development, as well as induces the expression of genes in nongonadal tissues that are necessary for oocyte growth. For instance, in the liver estradiol induce the production of choriogenin, a zona radiata precursor protein used in the egg envelope, and vitellogenin, a phospholipoprotein complex required for oocyte growth and used for larval energy supplies (Inui *et al.* 2003, Ng & Idler 1983). Vtg is transported to the ovary via blood, incorporated into the growing oocytes by micropinocytosis and cleaved into the proteins phosphovitin and lipovitellin (Wallace & Selman 1990, Ng & Idler 1983). Levels of vitellogenin in juvenile or male fish are normally low. However, on exposure to estrogens both estrogen receptors and Vtg genes are rapidly activated in the liver (Islinger *et al*. 2003). Hence, measurement of Vtg concentrations in juvenile and male fish has become a widely used biomarker for detecting estrogenic exposure. Different enzyme-linked immunosorbent assays (ELISAs) have been developed to quantifiy Vtg in zebrafish. These are based on the use of polyclonal antibodies against zebrafish vitellogenin (Fenske *et al*. 2001, Brion *et al*. 2002) or lipovitellin (Holbech *et al*. 2001), or a combination of monoclonal and polyclonal antibodies against vitellogenin (Nilsen *et al*. 2004). Zebrafish Vtg has been measured in various organs, including blood plasma (Fenske *et al*. 2001) and in homogenates of liver (Islinger *et al*. 2003) and whole-body fish (Holbech *et al*. 2001). In juvenile and adult zebrafish the lowest concentration of EE2 for inducing increased vitellogenin production is in the range of 1-10 ng/L (nominal concentration). In juvenile zebrafish elevated Vtg levels have been measured after exposure to 10 and 25 ng/L of EE2 (Hill & Janz 2003, Van den Belt *et al*. 2003). Increased Vtg concentrations were also observed in some individual fish exposed to 1 ng/L (Van den Belt *et al*. 2003). In adult male zebrafish a concentration dependent production of plasma Vtg at EE2 concentrations between 1.67-20 ng/L was measured (Fenske *et al*. 2001). In short-term studies with adult male zebrafish, increased Vtg production have been observed after exposure to 2.5, 4 and 5 ng/L of EE2 (Islinger *et al*. 2003, Rose *et al*. 2002, Kime & Nash 1999) with lack of induction at 0.85 and 2 ng/L of EE2 (Islinger *et al*. 2003, Rose *et al*. 2002).

Apart from responses on Vtg, phenotypic feminisation has also been observed after exposure of developing zebrafish to EE2. Juvenile zebrafish exposed to 3 and 10 ng/L of EE2 between days 43-71 post-fertilisation resulted in all-female stocks, whereas exposure to 1.67 ng/L of EE2 resulted in 85% females (Maack & Segner 2004). Female-biased sex ratios at exposures of juvenile zebrafish to EE2 concentrations ≥10 ng/L have also been observed by others (Hill & Janz 2003, Van den Belt *et al*. 2003). Feminisation have been indicated on exposure of juvenile zebrafish to 0.1 and 1 ng/L of EE2, although no statistics were used and large numbers (40%) of fish could not be sex determined (Van den Belt *et al*. 2003). Effects on secondary sex characteristic in zebrafish have also been observed on estrogenic exposure. In adult zebrafish, the presence of well-developed uro-genital papillae in mature females can be used to distinguish females from males. Exposures of adult males to E2 have been shown to induce the outgrowth of the uro-genital papillae (Brion *et al*. 2004). In comparison with

other fish species, such as the Japanese medaka, exposure to estrogens seems not to cause significant occurrences of gonadal intersex in zebrafish. Intersex have been observed when zebrafish were exposed to EE2 during different time windows from hatch to 60 days post-hatch, although at low ratios (Andersen *et al*. 2003). Other effects on estrogen exposure include a negative impact on oocyte maturation and reproduction in females (Hill & Janz 2003, Van den Belt *et al*. 2003, Van der Ven *et al*. 2003, Maack & Segner 2004) and inhibited spermatogenesis in males (Van den Belt *et al*. 2002, Van der Ven *et al*. 2003, Ortiz-Zarragoitia & Cajaraville 2005).

In zebrafish, vitellogenin measurement in connection with exposure to androgens have not been studied to the same extent as that of exposure to estrogens. In adult male zebrafish no decreases in Vtg levels were measured when fish were exposed for 7 days to nominal 17α-methyltestosterone (MT) concentrations between 2.5-100 ng/L (Andersen *et al*. 2006). Exposure of zebrafish to androgens can result in skewed sex ratios towards both males and females, depending on the type of androgen and dosage. Feminisation after exposure to androgens is probably due to aromatisation of the androgen into an estrogen, e.g. aromatisation of 17α-methyltestosterone into 17α-methylestradiol (Hornung *et al*. 2004). All-male stocks have been observed after exposure of juvenile zebrafish to 0.1 and 1 µg/L of 17α-methyldihydrotestosterone (MDHT; Wester *et al*. 2003). Exposure of juvenile zebrafish to the aromatisible MT from fertilisation to sexual maturation resulted in shift in sex ratios, with male-biased sex ratios observed at 1 µg/L and female-biased sex ratios at 10 µg/L (Andersen *et al*. 2001). Moreover, complete gonadal feminisation has been observed after exposure of juvenile zebrafish to 10 µg/L of MT (Fenske & Segner 2004). Exposure to MT can also result in gonadal intersex (Andersen *et al*. 2001). Other effects observed in zebrafish on androgen exposure include inhibited vitellogenesis of oocytes and decreased size of previtellogenic oocytes in females, as well as Sertoli cell hypertrophy and hyperplasia in males after exposure to 1-100 µg/L of MDHT (Van der Ven *et al*. 2003).

Apart from androgens, aromatase inhibitors have also been reported to cause masculinisation in zebrafish. Male-biased sex ratios were observed when juvenile zebrafish were exposed to 0.1-100 ng/L of tributyltin (McAllister & Kime 2003). Masculinisations have also been reported after exposure of juvenile zebrafish to 10-1000 µg/g of the aromatase inhibitor Fadrozole in the feed (Fenske & Segner 2004, Uchida *et al*. 2004). When juvenile zebrafish were exposed to Fadrozole via water (10-100 µg/L) the number of females was reduced and more fish with undifferentiated gonads were detected, but no increase of males was observed (Andersen *et al*. 2004). The same findings were observed when juvenile zebrafish were exposed to ZM 189,156 which acts as an estrogen receptor antagonist (Andersen *et al*. 2004). Moreover, both Fadrozole and ZM 189,156 exposures caused small increases in Vtg concentrations (Andersen *et al*. 2004).

Aims of the study

The overall aim was to develop a partial life-cycle zebrafish test for risk assessment of EDC.

The specific objectives were to:

Describe the period of gonad transformation under prevailing environmental laboratory conditions, and evaluate if exposures to estrogen and androgen during the juvenile phase affects gonad development and Vtg production.

Compare the sensitivity of the endpoints Vtg and gonad development after exposures to estrogen and androgen in zebrafish and Japanese medaka; two species suggested by OECD for risk assessment of EDC.

Evaluate estrogenic and androgenic effects in zebrafish exposed to binary mixtures of differently acting substances.

Evaluate if the partial life-cycle test can be used for risk assessment of effluents containing EDC.

Material and methods

Fish and chemicals

Adult zebrafish (*Danio rerio*) were bought from a local supplier and adapted to laboratory conditions for 1-2 months prior to the studies (Papers I-IV). The Japanese medaka (*Oryzias latipes*) (Paper II) were kindly provided by University of Hokkaido, Japan. The studies were performed at the Department of Biomedical Sciences and Veterinary Public Health, SLU. Zebrafish and medaka were held in a temperated lab at 26±1°C and a 12-h light/dark cycle. In the experimental studies, fish were held in standardised water (ISO 7346-1, 1996) (Papers I, III and IV) or charcoal filtered tap water (Paper II). Effluent pulp mill water (PME; Paper IV) was sampled in February 2001 after the final activated sludge treatment step, immediately frozen and stored at -20°C. The water was thawed overnight in room temperature prior to use. Breeding of zebrafish took place in stainless steel funnels. Phenotypic females (n=5-10) and males (n=10-20) were selected and placed together. A few hours after onset of light in the morning, fertilised eggs were collected and transferred from the funnels either directly into the exposure tanks (Paper II) or into 10-20 L raising tanks (Paper I, III, IV). From adult female medaka, clusters of eggs were collected with forceps. Newly fertilised eggs were transferred directly into the exposure tanks (Paper II). Fish larvae were fed Sera micron (Sera®), Tetra AZ 100 (Tetra®), Artemia nauplii and powdered freeze-dried red grubs (Nutrafin®) three times daily. Juvenile and adult fish were fed commercial flake food (Sera®, Tetra®) and freeze-dried red grubs 2-3 times daily. The chemicals used were 17α-ethinylestradiol (EE2: Paper I; INC Biomedicals Inc. Ohio, USA and Paper II&III; Sigma-Aldrich Sweden AB), 17α-methyltestosterone (MT: Sigma-Aldrich Sweden AB), 17β-trenbolone (Tb: Sigma-Aldrich Sweden AB). Stock solutions of chemicals were made in ethanol (EtOH: Paper I) or methanol (MeOH: Paper II&III). The chemical solutions were pipetted into the water of the exposure tanks. Solvent concentrations in the tanks were less than 0.1%.

Experimental regimes

Semi-static exposure regimes were used in all studies, based on 50 % (v/v) renewal of water three times per week. At 38 days post-hatch 5-10 fish were sampled from each replicate tank, frozen in liquid nitrogen and analysed for whole-body vitellogenin concentrations. The remaining fish were sampled at termination of the experiments (60 days post-hatch). After euthanisation in MS222 (1 g/L) the fish were fixed and processed for histological evaluation of gonad morphology and sex determination.

The different exposure periods and specific exceptions in each of the studies were:

Paper I
Non-exposed zebrafish were regularly and randomly sampled between days 20-60 post-hatch from a 10-L stock tank and the gonadogenesis was studied.

Juvenile zebrafish were exposed between days 20-60 post-hatch to EE2 (1, 2, 5, 10, 25 ng/L) and MT (26, 50, 100, 260, 500, 1000 ng/L). Three replicate 4.5-L tanks per exposure concentration of EE2 and two controls were used. Each replicate started with 40 fish. No replicates were used in the MT study. Each dose started with 60 fish. At termination of exposure 20 fish were sampled for whole-body Vtg measurements.

Paper II
Juvenile zebrafish and medaka were exposed between days 1-60 post-hatch to Tb (10 and 50 ng/L) and EE2 (10 and 100 ng/L). Duplicate 10-L tanks per exposure concentration and controls were used. Each replicate started with 60 fish.

Paper III
Juvenile zebrafish were exposed between days 20-60 post-hatch to binary mixtures of Tb (1, 10, 50 ng/L) and EE2 (2 and 5 ng/L). Three replicate 10-L tanks per concentration including water controls and solvent controls were used. Each replicate started with 50 fish. Also single tanks with 2 or 5 ng/L of EE2 or 50 ng/L of Tb were used.

Paper IV
Juvenile zebrafish were exposed between days 10-38 days post-hatch to different dilutions of PME (0.67, 2.5, 10 and 50% v/v). Three replicate 10-L tanks per pulp mill dilution and controls were used. Each replicate started with 50 fish.

Vitellogenin measurements

The sampled fish were prepared for Vtg analyses according to Holbech *et al.* (2001). Whole-body Vtg concentrations were measured using a direct non-competitive sandwich ELISA described by Holbech *et al*. (2001), or by using commercial zebrafish and medaka Vtg ELISA kits (Biosense laboratories®, Norway).

Histological preparations

Non-exposed juvenile zebrafish sampled for studies of gonadogenesis (Paper I) were fixed in a mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The fish were post-fixed in phosphate buffered 1% $Os0_4$ (Analytical Standards AB, Sweden), dehydrated in a graded series of ethanol, followed by immersion in 1-2-propylene oxide (Merck, Germany) and finally embedded in Agar 100 resin (Agar, U.K.). Semi-thin sections were cut with a glass knife, stained with toluidine blue and examined by light microscopy (LM). Some of the specimens were further evaluated using transmission electron microscopy (TEM). Ultra-thin sections were cut with a diamond knife, double stained with uranyl acetate and lead citrate and examined in a Philips TEM 420.

Fish exposed to steroids (EE2, MT, Tb) and pulp mill effluent, including controls, were fixed *in toto* in either Lillies Neutral Fixative (Paper I) or neutral buffered 10% formalin (Papers II-IV). The specimens were dehydrated using a graded

series of ethanol, treated with xylene and 6-10 individuals embedded in each paraffin block. The blocks were sectioned longitudinally in a dorsal-ventral position. Approximately 10-15 sections were cut in the gonadal region of the fish. The sections were stained with haematoxylin-eosin (HE) and examined by light LM.

Histological evaluation of gonads

LM evaluation was used for classification of the maturity of the female and male gonads (Papers I & III). The maturation of ovaries was classified according to the oocyte maturation stages described by Selman *et al*. (1993). The maturation classification of testes was based on the presence and amount of spermatozoa in the tubular lumen. The maturity of the gonads was also evaluated using image analysis (Papers II & IV). Using digitized images of the gonad sections, areas of immature oocytes in females and areas of spermatozoa in males were selected by thresholding of the Red-Green-Blue values. The selected gonad areas were measured and compared with the total sectioned area of the gonad.

Statistics

Differences in Vtg concentrations between groups were analysed using the non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Dunn's test (Papers I&IV), or the non-parametric Mann-Whitney *U* test (Paper II&III). Fishers Exact Test was used to test differences in sex ratios and intersex ratios between controls and exposure groups (Papers I-IV). Measurement of areas in gonads using image analysis was tested using one-way ANOVA followed by the parametric Dunnet's test (Papers II&IV). The significance level was set at 0.95 ($p \leq 0.05$). The symbols *, **, and *** represent p-values of $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$.

Results

Gonad transformation in non-exposed zebrafish (Paper I)

Zebrafish at the age of 3-4 weeks post-hatch (wph) all had well-defined ovaries, with oocytes reaching the perinucleolar stage. In fish sampled during 4-5 wph, gross morphological changes with degeneration of the oocytes were observed. Oocytes were characterised by irregularity in size, and absence of nucleoli or presence of a single large nucleolus. Granulation and large vacuoles were apparent in the cytoplasm. During 5-6 wph the number of oocytes was reduced accompanied with an ingrowth of stromal cells. Nests with germ cells were present in the gonads. The earliest well-defined testis, containing late spermatids, was observed at 6 wph (40 days post-hatch).

Exposure to steroids (Papers I-III)

Generally, low mortalities were recorded when fish were exposed to steroids. The exceptation was exposure of zebrafish to 100 ng/L of EE2, which resulted in 100% mortality (Paper II).

Vitellogenin measurements

Exposure to 1-25 ng/L of EE2 (Paper I) resulted in a dose-dependent increase in Vtg concentrations at nominal concentrations ≥2ng/L. In Paper III, increased Vtg concentrations were measured after exposure to 5 ng/L of EE2, but not after exposure to 2 ng/L. Exposure to 10 ng/L of EE2 resulted in increased Vtg concentrations in zebrafish, but not in medaka (Paper II). Exposure of medaka to 100 ng/L of EE2 resulted in increased Vtg concentrations, although at a level less than in zebrafish exposed to 10 ng/L. Exposure to 26-1000 ng/L of MT (Paper I) resulted in decreased Vtg levels at the concentrations 100, 260, and 500 ng/L of MT. In fish exposed to 1000 ng/L of MT increased Vtg concentrations were measured when compared with fish exposed between 50-500 ng/L, but not when compared with control fish. Exposures to 50 ng/L of Tb, but not 10 ng/L, caused decreased Vtg levels in both zebrafish and medaka (Paper II). No effects on Vtg were observed when zebrafish were exposed to 50 ng/L of Tb in Paper III. Exposures to binary mixtures of 2 ng/L of EE2 combined with 10 or 50 ng/L of Tb resulted in decreased Vtg concentrations (Paper III). Increased Vtg concentrations were measured when zebrafish were exposed to 5 ng/L of EE2 in combination with either 1 or 10 ng/L of Tb (Paper III).

Gonad development

Sex ratio

The sex ratios of control fish varied in the different studies. The mean ratios of females recorded in the control groups were 52% (EE2 study, Paper I), 67% (MT study, Paper I), 67% (zebrafish, Paper II), 54% (medaka, Paper II), 59% and 66% (Paper III), and 51% (Paper IV).

Exposure of zebrafish to 1-25 ng/L of EE2 (Paper I) resulted in significantly more females in all concentrations. In groups exposed to 2, 5 and 10 ng/L of EE2 no males were observed. Exposure to 1 ng/L resulted in <10% males. However, 22% males were observed after exposure to 25 ng/L of EE2. In Paper III, exposures to 2 and 5 ng/L of EE2 resulted in 80% and 100% females. In medaka, no effect on sex ratio was observed after exposure to 10 ng/l of EE2, whereas in zebrafish 100% females were recorded (Paper II). Exposure of medaka to 100 ng/l of EE2 resulted in 88% females, 2% males, and 10% intersex fish. No intersex zebrafish were observed after exposure to EE2. After exposure to MT (26-1000 ng/L) no females were observed in any of the concentrations used (Paper I). Exposures to 260 and 1000 ng/L of MT resulted in 3% and 27% intersex fish, respectively. No effects on sex ratios were observed in either zebrafish or medaka after exposure to 10 ng/L of Tb (Paper II). Exposure to 50 ng/L of Tb resulted in 100 % males in zebrafish (Paper II, III), but no effect on sex ratio were observed in medaka. No intersex fish were observed after exposure to Tb. More males were detected after exposures to binary mixtures of 2 ng/L of EE2 in combination with 10 or 50 ng/L of Tb (Paper III). Exposures to 5 ng/L of EE2 in combination with 1 or 10 ng/L of Tb resulted in all-female stocks. Mixtures of 5 ng/L of EE2 with 50 ng/L of Tb resulted in 90% females. Intersex fish were observed after exposure to 2 ng/L of EE2 combined with 50 ng/L of Tb.

Gonad maturation

Females

Exposure to EE2 resulted in decreased maturity of oocytes (Papers I-III).
In Paper I and II, control female ovaries contained mainly immature oocytes, with a small proportion being classified as maturing. Exposures to 2, 5 and 10 ng/L of EE2 caused increased immaturity of oocytes, with none of the fish having maturing oocytes (Papers I, II). Increased ratios of immature oocytes were also observed in Paper III after exposure to 5 ng/L of EE2, compared with control females. No effect was observed after exposure to 2 ng/L (Paper III). Exposure of medaka to 100 ng/l of EE2 resulted in a higher proportion of more mature oocytes (6%), as compared with <1% in control females (Paper II). In females exposed to mixtures of EE2 and Tb generally more immature oocytes were present in the ovaries compared with controls (Paper III). However, relatively, more mature oocytes with increasing doses of Tb were observed.

Males

In zebrafish exposed to 26 and 50 ng/L of MT the testis of most males were classified as immature, as in control males (Paper I). Exposure to ≥100 ng/L of MT resulted in increased maturity of males. In both zebrafish and medaka, exposure to 50 ng/L of Tb resulted in increased testis area, as well as spermatozoa area (Paper II). Similar findings were observed in Paper III, with relatively more mature males detected after exposure to 50 ng/L of Tb. Compared with controls, less mature males were observed after exposure to 2 ng/L of EE2 mixed with 1 ng/L of Tb (Paper III). However, with increasing doses of Tb (10 and 50 ng/L) more mature males were observed.

Exposure to pulp mill effluent (Paper IV)

The mortality was high in all groups of fish, including controls. Apart from fish exposed to 50% PME, having 50% mortality, the mortalities were 30% in all groups.

Vitellogenin measurements

No significant differences in Vtg concentrations were detected in fish exposed to 0.67, 2.5 and 10% PME. Elevated Vtg concentrations were measured in fish exposed to 50% PME.

Gonad development

Exposure to PME resulted in a dose-related increase in the number of males. The mean percentage of males in the controls was 49%. The mean percentages of males in groups exposed to 0.67, 2.5 and 10% PME were 54%, 55% and 57%, respectively. Significantly ($p<0.05$) more males (mean 63%) was recorded after exposure to 50% PME.

A trend of increased area of immature oocytes, from 49% in controls up to 60% in fish exposed to 10% pulp mill effluent, was observed in females. In males the area of spermatozoa increased from 2.4% in controls to 3.3% in fish exposed to 50% PME.

General discussion

Gonad development in zebrafish

Gonad differentiation is a labile process and many factors can influence the development of the phenotypic sex. In gonochoristic fish species, the estrogen/androgen ratio has been suggested as the natural system for gonad differentiation (Baroiller & D'Cotta 2001). One mechanism regulating this ratio involves the cytochrome P450 aromatase enzymes, converting androgens to estrogens. In zebrafish two different aromatase genes have been described, one gonadal (Cyp19a) and one brain form (Cyp19b). Two different gene expression populations of the brain form were measured in zebrafish sampled between 24 to 40 days post-fertilisation, i.e. low (male) and high (female) expressors (Trant *et al*. 2001). These differential expression patterns indicate sex-specific gonadal differentiation in zebrafish between 3-5 weeks post-hatch. However, variations in the timing of gonad differentiation have been observed between different studies. Both Takahashi (1977) and Uchida *et al*. (2002) reported similar main periods of gonad transformation of ovaries into testes, with degeneration of oocytes starting at 3 weeks post-hatch (wph) and the disappearance of all oocytes at 4 wph. In the present study, this transformation period was more extended and mainly occurred between 4-5 wph. Similar findings were reported by Maack & Segner (2003), with transformation taking place approximately between 5-7 wph. Incomplete gonad differentiation in males at the age of 5-6 wph was also reported by Brion *et al*. (2004). Further studies by Maack *et al*. (2003) revealed no differences in time of gonad differentiation between different strains of zebrafish, nor was the differentiation size-dependent. The observed variations in the time of sexual development might be due to differences in biotic factors (e.g. stocking density, social behaviour and nutrition) or abiotic factors (e.g. pH, temperature and light regime). For instance, both a higher water temperature (28.5ºC vs 26ºC) and a longer light period (14 h vs 12 h) were used in the study by Uchida *et al*. (2002) compared with both the present study and the studies by Maack & Segner (2003) and Maack *et al*. (2003). In the study by Brion *et al*. (2004), a lower water temperature (25ºC) and a longer light period (15:9 h) were used.

The sex ratios of non-exposed zebrafish do not always display ratios of 1:1. In the present study, the ratio of females in the different studies varied between 51-67%. In other studies, control female ratios ranging between 24-72% have been reported (Andersen *et al*. 2003, Hill & Janz 2003, Van den Belt *et al*. 2003, Andersen *et al*. 2004, Brion *et al*. 2004, Fenske & Segner 2004, Maack & Segner 2004, Santos *et al*. 2006). Variations in sex ratios support the findings that the zebrafish lack sex chromosomes and thus suggest polygenic sex determination or influence of environmental factors, or combinations of both (Devlin & Nagahama 2002). However, until the sex determining factors in zebrafish are fully understood and no genetic sex markers exist, care must be taken when using sex ratio as an endpoint. The cause to inter-study variations in sex ratios might be that sex is determined solely by environmental factors or that environmental factors can override the sex determining genes. Thus, the observed phenotypic sex of an adult

fish might not necessarily reflect the genetic sex. Although fish are kept at constant environmental laboratory conditions, small differences in the conditions might be important and play decisive roles in sexual differentiation. Another possible reason for variations in sex ratios is sex dependent early life stage mortality. If mortality occurs predominantly among one of the sexes during early development, an excess of the opposite sex already exists when the individuals are selected for studies. The main disadvantage using fish with non-stable sex ratios is that deviations are not revealed until fish are sexed at the end of the study. This also means that at large deviations the results might have to be discarded and the experiment repeated. The advantage is that sex differentiation might function as a highly sensitive endpoint when evaluating substances with endocrine activity. This has been indicated in the present study, where sex ratio seems to be an equally or even more sensitive biomarker than measurement of Vtg production. In juvenile zebrafish whole-body Vtg concentrations are similar in fish at the age of 25, 32 and 39 dph (Andersen *et al*. 2003). However, somewhat increased Vtg levels were measured in fish sampled at 46 dph, indicating start of vitellogenesis in some individuals (Andersen *et al*. 2003). In the present study, measurement of Vtg in non-exposed fish at the age of 38 dph resulted in homogenous data with low variation. Thus, measurement of Vtg in juvenile zebrafish can be recommended in fish at the age of approximately 40 dph, since the vitellogenesis still is low.

Exposure to steroids and pulp mill effluent

Three different exposure periods were used in the present study, i.e. 20-60 dph, hatch-60 dph, and 10-38 dph. Exposure during all these three periods affected Vtg production and gonad development. The timing of exposure to EDC during zebrafish development can affect the sex ratios differently. Exposure of zebrafish to 15 ng/L of EE2 during different time periods from hatch to 60 dph revealed certain critical time windows for successful reversal of the sex (Andersen *et al.* 2003). Complete sex reversal was observed when exposure was conducted between hatch and 60 dph, and between 20 and 60 dph. Female-biased sex ratios were also observed when fish were exposed between 10-20 dph and 20-40 dph although these periods also resulted in intersex fish.

The present study showed that exposure during 20-38 dph to nominal EE2 concentrations between 2-25 ng/L results in dose-dependent increases in whole-body Vtg. The response on Vtg production seems similar in juveniles and adult male zebrafish. Adult males exposed for 21 days resulted in dose-dependent increases of plasma Vtg at nominal EE2 concentrations between 1.67-20 ng/L (Fenske *et al*. 2001). Moreover, exposure of juvenile zebrafish between 20-60 dph to 1-25 ng/L of EE2 resulted in female-biased sex ratios at all tested concentrations. Similar results were observed when juvenile zebrafish were exposed to 3 and 10 ng/L of EE2 between days 43-71 post-fertilisation, resulting in all-female stocks, whereas exposure to 1.67 ng/L of EE2 resulted in 85% females (Maack & Segner 2004). The potency of EE2 is reflected in the narrow response ranges observed. Small differences in actual exposure concentrations might explain deviations in responses of both Vtg production and sex ratios

between the different studies. For instance, whereas exposure to 2 ng/L of EE2 resulted in 100% females in Paper I, the same concentration resulted in 80% females in Paper III. Moreover, exposure to 5 ng/L of EE2 in Paper I resulted in highly elevated Vtg levels, whereas in Paper III moderately increased levels were measured. The potency of EE2 as a very strong estrogen was also revealed when mixed with Tb. A few ng/L of EE2 resulted in female-biased sex ratios despite the presence of an androgen at higher concentrations. These findings implicate that the effects of EDC on the population level might be more dependent on the concentration of EE2 than that of androgens. Since EE2 is not uncommonly detected at low ng/L concentrations, this might pose serious threats to wild fish living in waters contaminated with strong estrogens, such as EE2.

Exposure to EE2 has in many studies been demonstrated to negatively affect gonad maturation in zebrafish. In the present study, exposure to EE2 resulted in reductions of mature oocytes and large proportions of fish with undifferentiated gonads. Similar findings have also been reported in other studies, together with atresia and reduced ovary-somatic index after exposure to estrogens (Hill & Janz 2003, Van den Belt *et al*. 2003, Van der Ven *et al*. 2003, Maack & Segner 2004). Obviously normal phenotypic females can develop after exposure to EE2. However, several negative effects on reproduction have been observed, such as delayed or inhibited onset of spawning, reduced number of spawning females, reduced egg production, as well as reductions in fertilisation and hatchability of the eggs (Hill & Janz 2003, Van den Belt *et al*. 2003, Maack & Segner 2004). Moreover, zebrafish male reproduction can be affected by estrogens, i.e. inhibited spermatogenesis (Van den Belt *et al*. 2002, Van der Ven *et al*. 2003, Ortiz-Zarragoitia & Cajaraville 2005). However, many of the effects have been demonstrated to be reversible. Recovery periods after estrogenic exposure have revealed outgrowth and maturation of both testes and ovaries, as well as normalised sex ratios (Hill & Janz 2003, Van den Belt *et al*. 2003, Maack & Segner 2004).

Exposure to the androgenic steroids MT and Tb resulted in decreased Vtg concentrations, as well as skewed sex ratios towards males. Comparisons of MT and Tb indicate similar response levels although different exposure periods and durations were used. In zebrafish, effects on Vtg in connection with exposure to androgens have not been studied to the same extent as after exposure to estrogens. In the present study, exposures to 100-500 ng MT/L and 50 ng Tb/L resulted in decreased Vtg concentrations. No decreases in Vtg levels were measured when adult male zebrafish were exposed to MT concentrations between 2.5-100 ng/L (Andersen *et al*. 2006). However, this lack of reduced Vtg levels might be explained by the shorter exposure period used (7 days) compared with the present study. In female fathead minnows decreased Vtg concentrations have also been measured after exposures to androgens, i.e. 10 µg/L of dihydrotestosterone (DHT) and ≥50 ng/L of Tb (Ankley *et al*. 2003, Panter *et al*. 2004). Apart from reducing the Vtg levels, exposures to high androgen concentrations can cause increased production of Vtg. This was indicated in the present study, and has also been observed in other exposure studies using the aromatisible MT (Pawlowski *et al*. 2004, Hornung *et al*. 2004, Zerulla *et al*. 2002). In adult male fathead minnows,

MT was shown to be converted into 17α-methylestradiol to levels equal that of E2 in adult females, and induce the production of Vtg (Hornung *et al*. 2004). However, increased Vtg levels might also be due to other mechanisms than direct aromatisation of exogenous androgens, such as binding and activation through the estrogen receptor, or indirectly by triggering aromatisation of endogenous androgens. In male fathead minnows increased Vtg concentrations have been measured after exposure to non-aromatisible androgens, such as 10-100 µg/L of DHT (Panter *et al.* 2004) and 50 µg/L of Tb (Ankley *et al*. 2003). Low-affinity binding of Tb to the fathead minnow estrogen receptor was suggested to cause to the increased Vtg levels (Ankley *et al*. 2003). Low-affinity binding of Tb to the estrogen receptor of rainbow trout has also been observed (Le Guevel & Pakdel 2001).

Exposure of zebrafish to androgens can result in skewed sex ratios towards both males and females, depending on the properties and concentration of the androgen. In the present study, both exposures to MT (26-1000 ng/L) and Tb (50 ng/L) resulted in male-biased sex ratios, including all-male stocks. All-male stocks have also been observed when juvenile zebrafish were exposed to 0.1 and 1 µg/L MDHT (Wester *et al*. 2003). Male-biased sex ratios have previously been observed when juvenile zebrafish were exposed to 1 µg/L of MT (Andersen *et al*. 2001). However, exposure to 10 µg/L resulted in female-biased sex ratios (Andersen *et al*. 2001). Moreover, complete gonadal feminisation has been observed after exposure of juvenile zebrafish to 10 µg/L of MT (Fenske & Segner 2004). Feminisation after exposure to androgens is probably mainly caused by aromatisation of the androgen into an estrogen, such as the conversion of MT into 17α-methylestradiol (Hornung *et al*. 2004). Exposure to Tb has also in other fish species been reported to cause masculinisation. In female fathead minnows, induction of male-specific phenotypic secondary sex characteristics, i.e. dorsal nuptial tubercles, was observed after exposure to Tb concentrations ≥50 ng/L (Ankley *et al*. 2003). Exposures to DHT (10-100 µg/L) and MT (1-50µg/L) also increased nuptial tubercles in fathead minnows (Panter *et al*. 2004, Pawlowski *et al*. 2004). Moreover, phenotypic masculinisation, i.e. induced outgrowth of the anal fin gonopodium, which is used as a copulatory organ, was observed in both juvenile and adult female mosquitofish (*Gambusia affinis*) after exposure to Tb concentrations between 0.3-10 µg/L (Sone *et al*. 2005). Apart from that exposure to both MT and Tb resulted in male-biased sex ratios in the present study both androgens also stimulated the maturation of the testis, with increased testicular area and enhanced spermatogenesis. Stimulated spermatogenesis has also been observed after exposure of juvenile zebrafish to MDHT (Wester *et al*. 2003).

The male-biased sex ratios in zebrafish observed after exposure to PME confirm previous studies indicating the presence of androgenic substances. In a recent paper by Larsson *et al*. (2006) chemically fractionated extracts of effluent water from the same pulp mill was tested in a competitive androgen receptor binding assay from ovaries of Atlantic croaker (*Micropogonias undulates*). The primary effluent contained 96 dihydrotestosterone equivalents/L and the final effluent 6 ng/L. Further chemical analyses revealed 35 androgen receptor ligands in different fractions, supporting the notion that masculinisation is caused by androgens

present in the PME (Larsson *et al*. 2006). The increased levels of Vtg observed in zebrafish might be due to aromatisation of the androgens, or simultaneous presence of androgens and estrogen receptor ligands, or both. Indications of effects on secondary sex characteristics due to aromatisation of androgens have also been observed in fathead minnows exposed to bleached sulfite mill effluents. Exposure to lower effluent concentrations resulted in masculinisation of females, whereas feminisation of males was observed at higher concentrations (Parrot *et al*. 2003).

In zebrafish, prolonged exposure up to 60 dph to EE2 seems not to induce any significant development of intersex gonads, which is in agreement with the results of Andersen *et al*. (2003). However, short periods of exposure to EE2 during sexual development induce intersex (Andersen *et al*. 2003). In the present study, exposure to 1 µg/L MT resulted in a large ratio of intersex zebrafish, which is similar to the findings of Andersen *et al*. (2001). Moreover, intersex was also observed when zebrafish were exposed to PME and to mixtures of EE2 and Tb. This disruption in gonad development might result from sudden changes in hormonal milieu, which can occur during short-term exposure windows or when aromatisation of androgens starts during gonadal development. In three-spined sticklebacks, genetic sex identification is possible through male sex-linked DNA markers (Hahlbeck *et al*. 2004). In sticklebacks exposed to E2 (1 and 10 µg/L) and EE2 (50 ng/L) all fish that developed intersex were identified as genetic males (Hahlbeck *et al*. 2004). In sticklebacks exposed to 1 µg/L MT from hatch to 14 days, all genetic females and most males developed testis (Hahlbeck *et al*. 2004). In sticklebacks exposed from 14 days to end of experiment (day 39-58), the genetic females developed ovaries and all males were partially sterile. Moreover, continuous exposure from hatch to end of experiment resulted in all the genetic females developing intersex, and most genetic males were partially sterile. The development of intersex in the genetic females was suggested to be due to re-feminisation of initially masculinised females, as a consequence of aromatisation of MT (Hahlbeck *et al*. 2004). The findings in sticklebacks might suggest that androgen exposure triggers intersex formation in females, and estrogen exposure triggers intersex in males.

Major conclusions

• Measurement of whole-body Vtg in juvenile zebrafish at 40 dph is recommended, since vitellogenesis in non-exposed fish is low and responses of exposure to EDC at relatively low concentrations can be detected. The Vtg levels can indicate the outcome of phenotypic sex in zebrafish exposed to EDC. However, elevated Vtg levels do not necessarily result in feminisation as seen in fish exposed to PME.

• Sex ratio at 60 dph is an appropriate endpoint to be used for evaluation of EDC, since it is equally or even more sensitive than Vtg, and also covers androgenic effects.

• Zebrafish exposed during the period of gonad differentiation are sensitive to single steroids, as well as complex mixtures of EDC. The tested endpoints Vtg and gonad development are suitable for studying estrogenic and androgenic effects. The combination of Vtg and sex ratio are recommended since they act at different biological levels covering both individual and population responses.

• The zebrafish respond to EDC at environmentally realistic concentrations, which support zebrafish as a suitable model species for environmental risk assessment and regulatory purposes.

Future perspectives

Since the mechanism of sex determination in zebrafish is unknown, possible environmental factors determining or influencing the phenotypic sex needs to be further studied. Moreover, the importance of environmental factors on the timing of gonad differentiation needs to be evaluated.

The response of zebrafish to substances with different modes of action, such as anti-estrogens, anti-androgens, and aromatase inhibitors needs to be further studied.

Wild fish are normally exposed to mixtures rather than to single substances. The effects of multi-component mixtures of EDC with similar modes of action need to be evaluated, and also whether the effects can be predicted from knowledge of the individual potencies.

Complementary tests based on zebrafish, including *in vitro* embryo assays and full life-cycle assays, should be introduced in order to cover additional effects caused by EDC at different biological levels.

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