JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
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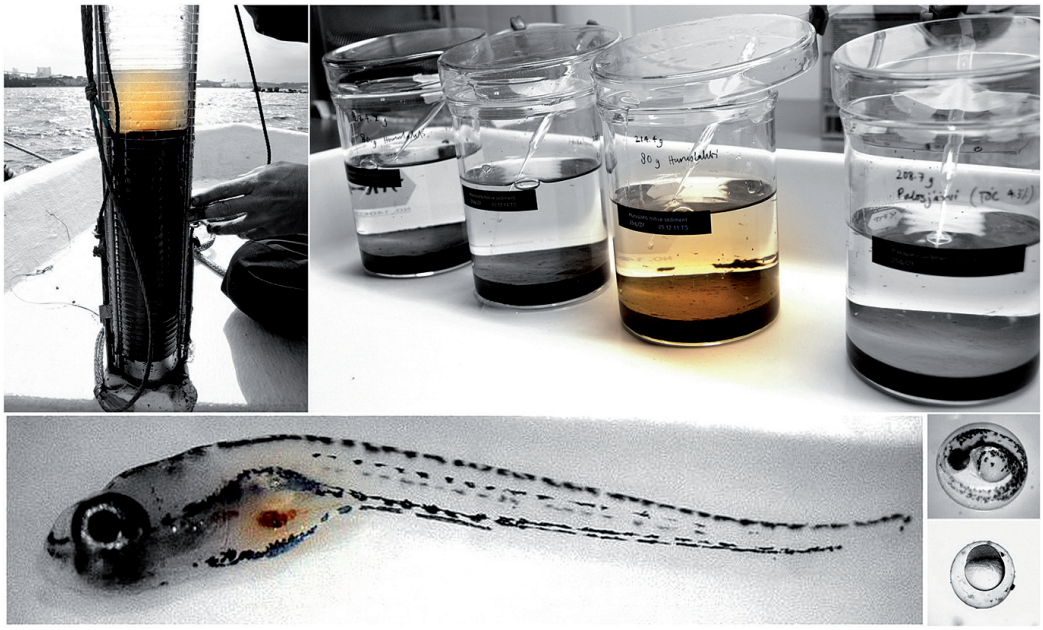

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ABSTRACT

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Advancing the ecotoxicological relevancy of zebrafish: Application of early-juvenile *20dpfZF* to assess xenoestrogenicity of environmental chemicals and samples
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Diss.

To assess xenoestrogenicity, the 20 days-post-fertilization zebrafish (*20dpfZF*) life-stage was experimentally exposed to known model chemicals as well as to environmentally contaminated samples. From a selection of key responsive genes, molecular responses associated with the steroidogenic impact (brain aromatase, *cyp19a1b* and vitellogenin isoform 1, *vtg1*) were used to assess the endocrinic modulation by xenoestrogenic samples. This work showed diverse *in vivo* responses by the *20dpfZF,* from pure chemicals (bisphenol A, BPA; 17α-ethinylestradiol, EE2; nonylphenol, NP; octylphenol, OP), and their mixture combinations, and to industrially contaminated sediment samples. Gene expression analysis of *cyp19a1b* and *vtg1* in exposed *20dpfZF* showed the estrogenic potencies of chemicals in the order: EE2 > OP > BPA > NP. Transcriptional up-regulation of *vtg1* in early juvenile fish exposed to mixtures (BPA + NP + OP), relative to individual concentrations, suggest in minimum the additive *in vivo* effect of a mixture of very low sublethal doses of single xenoestrogens. Complementary expression of *cyp19a1b* and *vtg1* in *20dpfZF*, exposed to artificially-spiked sediment with EE2 established the estrogen-responsiveness of the model. In contrast, the lack of simple estrogenic or anti-estrogenic trait of correspondence in biomarker transcription was evident from exposure to two types of industrially contaminated sediments, suggesting common non-linearity of *in vivo* response by the model. The results also highlight the applicability of *20dpfZF* in the assessment of remediation and monitoring of historically polluted sites. This study showed that the *20dpfZF* addressed the regulatory requirement of a multi-purpose and cost-effective ecotoxicological model capable of *in vivo* genomic assessment of endocrine-active substances in aquatic samples.

Keywords: aromatase; estrogenicity; *in vivo* early life-stage; vitellogenin; zebrafish.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals I–IV.

In all articles, I am the first author, as Sahoo, and contributed significantly to the planning, maintenance of animals, experimental data collection, analyses and writing. I have been the corresponding author in all articles (I–IV). In article II, Sillanpää as the second author also contributed to the experimentation, data collection and analyses.

I Sahoo T.P. & Oikari A. 2013. Use of early juvenile zebrafish *Danio rerio* for *in vivo* assessment of endocrine modulation by xenoestrogens. *Journal of Environmental and Analytical Toxicology* 4 (1): 202. doi: 10.4172/2161-0525.1000202

II Sahoo T.P., Sillanpää K., Lahti M., Vehniäinen E.-R., Heinonen T. & Oikari A. Modulation of estrogen-responsive gene induction by xenoestrogenic mixtures in early juvenile zebrafish. Submitted manuscript.

III Sahoo T.P. & Oikari A. Use of early life-stages of zebrafish to assess toxicity of sediments contaminated by organotin compounds. Submitted manuscript.

IV Sahoo T.P. & Oikari A. Probing the xenoestrogenic impact of sediment cores contaminated by the pulp and paper industry: Induction of aromatase *cyp19a1b* in early juvenile zebrafish. Submitted manuscript.

ABBREVIATIONS

20dpfZF	20 days post-fertilization old zebrafish
AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
AR	androgen receptor
BKME	bleached kraft mill effluent
BPA	bisphenol A
CA	concentration addition
CYP11A1	cytochrome P450 11A1
CYP19A1B	cytochrome P450 19A1B
CYP1A1	cytochrome P450 1A1
dpf	days-post-fertilization
dw	dry weight
E2	17β-estradiol
EDCs	endocrine disrupting chemicals
EE2	17α-ethinylestradiol
ELS	early life-stage
ER	estrogen receptor
ERE	estrogen responsive element
GC-MS	gas chromatography – mass spectrometry
hpf	hours-post-fertilization
HPG	hypothalamus pituitary gonad axis (+L, liver)
IA	independent action
LC-MS	liquid chromatography – mass spectrometry
log K_{ow}	logarithmic of octanol water partition coefficient
MeoA	mechanism-of-action
MoA	mode-of-action
NP	nonylphenol
OC	organic carbon
OP	octylphenol
OTC	organotin compounds
PPM	pulp and paper mill
qPCR	quantitative polymerase chain reaction
RA	resin acid
SD	standard deviation
TOC	total organic carbon
VTG	vitellogenin
Vtg1	vitellogenin 1
WS	wood sterol
ww	wet weight

1 INTRODUCTION

1.1 Chemicals in the environment

The worldwide production and circulation of chemicals, ranging from basic to industrial, speciality and consumer applications, is estimated to be annual worth over $3 trillion (Cefic Chemdata International 2013). In the European context, seven of the 28 member states make up 85 % of the total chemical sales in the European Union, the EU being the world's top trader of chemicals in 2012. Over the past decade, China and the rest of Asia, comprising largely developing markets, amounted to about a 30 and 20 per cent share of the global chemical sales respectively, highlighting the significance of the chemical industry for these emerging economies. Of particular relevance to environmental risk estimations, the current regulatory mechanisms are limited by the production, distribution and disposal of large volumes of novel chemicals that are deployed before regulations come into effect.

Anthropogenic point sources affect aquatic organisms living downstream of receiving water bodies. Synthetic compounds, many of which are structurally capable of accumulating and targeting an aquatic species, are being continuously introduced into the environment due to their extensive usage in daily applications. On the other hand, although ecotoxicology seeks to address effects on populations, via a broad ecological perspective, the identification of specific target organs and metabolic pathways in animals exposed to and affected by environmental chemicals, which occur in numerous combinations, is gaining in emphasis. In the European context, chemical control of point sources is under the preview of the European legislative guide for the registration, evaluation, authorisation and restriction of chemicals (REACH), due to the large production volumes of both industrial and daily-use chemicals, and their potential distribution in different environmental compartments (EU 2006). Specific attention has also been directed to the marketing of chemicals in the classification, labelling and packaging (CLP) of substances and mixtures (EU 2008a). To facilitate necessary risk assessment of chemicals, the OECD

(Organisation for Economic Co-operation and Development) testing guidelines defined for fish species (OECD 2012). They include threshold testing at assessment endpoints for acute toxicity (OECD 203 Fish acute toxicity test), animal growth (OECD 215 Fish, Juvenile growth test), bioconcentration (OECD 305 Flow-through fish test), and endocrine disruption (OECD 229 Fish short term reproduction assay) among other listed tests. These recommendations were developed in keeping with a more ethical approach to vertebrate testing through the application of the 3R principles – replacement, reduction and refinement, of experimental methods (EU 2010). In particular, *in vitro* research, despite its applicability is not without uncertainty, over-simplifies the complexity of *in vivo* responses as well as those emerging in populations.

The Fish Embryo Acute Toxicity Test (FET, OECD TG 236), with emphasis on early life-stage (ELS) of fish, has found acceptance from some scientific circles, but being carried out at an immobile and non-feeding life-stage presents discrepancies in addressing complex exposure scenarios i.e., egg-chorion permeability. Aquatic toxicity testing with a sensitive ELS, representative of a mobile adult that shows physiological sensitivity to exposure, may fit better within the improved toxicity testing framework. Upon risk management of toxic chemicals, their pre-control is implemented by the restricted production and end-usage of such chemicals, from an environmental safety perspective. Chemical contamination of various environmental media by other point discharges, include effluent from industrial and sewage treatment plants, emissions being exclusively mixtures are also under regulatory control, e.g. along with mitigation of the Directive on Industrial Emissions Integrated Pollution Prevention and Control (IED) (EU 2010). Control of such pollution is not addressed by chemical regulatory agencies, even though environmental samples come from a combination of pollutants from point and non-point sources that are contaminated or remediated sites. Of particular importance to surface aquatic ecosystem quality is the toxicity of deposited substances in sediment and their potential to contaminate the water column by remobilisation (Chapman *et al.* 2002, Eggleton and Thomas 2004).

Given that the research needs for a model test animal must be viewed from the ecological outlook, chemical accumulation and distribution through aquatic food webs generally indicate fish among top predators. The transfer or uptake of chemicals in fish accounts for accumulation via diet, besides integumental routes of exposure, and thereby fish models present excellent systems for incorporating pathways for chemical uptake, distribution, metabolism and subsequent effect at target organs (Kleinow *et al.* 2008). Furthermore, some biotransformation studies have revealed the toxicity of excreted metabolites to other organisms (Schlenk *et al.* 2008). Among vertebrate taxa, teleosts thus qualify as sensitive candidates to investigate chemical-mediated adverse responses (Kime 1998, Segner *et al.* 2003, Van den Belt *et al.* 2003, Kidd *et al.* 2007, Scholz and Mayer 2008).

When aquatic bodies receive chemicals and their mixtures, properties such as polarity, hydrophobicity, stability and presence of charged groups

contribute to their environmental fate. Persistence of chemicals in the environment can be attributed to their bioaccumulative potential. Bioaccumulation of aquatic pollutants is defined as the outcome of uptake of xenobiotics in the body of an organism, by contact with contaminated water, sediment and pore water in sediment, and the rate of uptake exceeding that of its removal from the body. The increase in concentration of a contaminant in the aquatic organism relative to the concentration in the ambient water, known as bioconcentration, is quantified as the bioconcentration factor (BCF). In this, hydrophobicity of an organic chemical, described by its octanol-water partition coefficient, log K_{ow}, is central to the assessment of its behavior in the various environmental compartments (Erickson *et al.* 2008). Further, dissolved organic matter (humic substances) cause significant reduction in bioavailability due to hydrophobic sorption of chemicals. Another critical factor contributing to bioavailability is the speciation of ionizable compounds, which is determined by both chemical-specific dissociation (pK_a) and by the specific pH of aquatic systems. In the regulatory context, chemical substances with log $K_{ow} \geq 3$ are assessed for their bioaccumulative potential (OECD 305 Bioaccumulation in Fish: aqueous and dietary exposure). Traditionally, chemicals of concern in aquatic systems include for example PAHs, PCBs, PCDFs, PCDDs and agro-chemicals, with large K_{ow} values (log $K_{ow} > 5$) which are categorized as highly hydrophobic compounds, and which strongly associate with fish gills and skin as well as to organic matter in sediments.

Once the xenobiotic chemical is bioavailable for uptake by fish, distribution, metabolism and elimination pathways within the organism determine the degree of internal dosage or exposure, which contributes to physiological effects. While it is challenging to fully define the effects-characterization of a chemical or its metabolite, work on the binding affinity of such ligands to biological molecular targets, has elucidated the implications of receptor-mediated responses in key biological functions such as endocrine signaling (Hahn and Hestermann 2008). Considering that endocrine signals can extensively regulate the reproductive status of an organism, and thereby have population-level repercussions, exogenous chemicals (ligands) that mimic naturally occurring hormones are among the "usual suspects" in the adverse effects of environmental contamination.

1.2 Endocrine modulation in fish by xenohormonal compounds

1.2.1 Impact of receptor-mediated actions in endocrine signalling

Chemicals from anthropogenic sources find their ways into freshwater and subsequently marine ecosystems (Erickson *et al.* 2008). Over the past few decades, owing to the industrial and consumptional pollutants entering the water bodies, environmental research has also concentrated on endocrine disrupting chemicals (EDCs) capable of altering hormonal status and leading

up to impaired reproduction in fish and other aquatic organisms (Baroiller *et al.* 1999, Hahn and Hestermann 2008). Of particular concern is the toxicity of low-dosage EDCs capable of acting individually or in combination and which induce diverse endocrine impacts (Brian *et al.* 2005). Developmental and physiological responses in aquatic vertebrates are coordinated by feedbacks and other regulatory mechanisms, over the neuroendocrine system (Devlin and Nagahama 2002, Hiramatsu *et al.* 2005) for example. Fish treated with natural or synthetic hormones as environmental factors can even experience sex reversal in synchronous hermaphroditic as well as in gonochoristic species, with male or female reproductive organs in different individuals (Baroiller *et al.* 1999).

A part of vertebrate endocrine signalling is based on the response to endogenous steroids (androgens and estrogens) that occupy receptors that initiate a cascade of processes, eventually leading to the activation of respective steroid-responsive genes prior to the production of phenotype-specific proteins (Hiramatsu *et al.* 2005) (Fig. 1). Since receptor-binding by the ligand (steroid) is central to the endocrine pathways, exogenously derived synthetic ligands, owing to their similar structure and potential for mimicking steroids, successfully outcompete physiologically endogenous steroids in androgen (AR) and estrogen (ER) receptor binding (Goksøyr 2006). These events compromise the normal functionality of the steroid biosynthetic pathways. In estrogenic signalling, following ligand binding to ERs, subsequent binding of ER-homodimers to estrogen-responsive elements (ERE) in the regulatory region (promoter) of susceptible genes initiate the transcription and translation of the estrogen-responsive egg-yolk precursor vitellogenin (Vtg). This may take place also in male individuals, though normally is found only in female oviparous organisms. In fish, such vital and diverse events in the hypothalamic-pituitary-gonadal (HPG) axis point to the relevance of finding key biomarkers indicative of disrupted fish endocrine systems that have been influenced by abnormal or altered endogenous hormonal concentrations, and what the effects mediated by that are (Nakamura *et al.* 1998, Devlin and Nagahama 2002).

Within the context of endocrine disruption, the HPG axis is extended to include the liver, the front site of vitellogenesis (Arukwe and Goksøyr 2003). Furthermore, the endocrine system in fish responds to the collective of signals provoked at different sites of chemical interferences, including sense organs (eyes, pineal, olfactory organs), brain, pituitarygland, gonads (ovary, testis) and liver (Thomas 2008).

In the steroid biosynthesis of fishes and other vertebrates, cholesterol is the precursor molecule, which is enzymatically converted to endogenous hormone products, testosterone (T) and 17β-estradiol (E2) via a series of biochemical reactions (Fig. 1). Of particular significance to the estrogenic response at the cellular level is the catalytic aromatization of C-19 androgens (T) into C-18 estrogens (E2) by the enzyme complex, cytochrome P450 aromatase (Cyp19). Subsequently, steroid biosynthesis is regulated by feedback control of sex steroids within gonads. Along these events, the identification of an early life-stage (ELS) of gonochoristic fish that is representative of a bipotential

gonadal and sex differentiation period, constitutes a relevant indicator in exposure tests. That such plasticity of gonadal development and differentiation takes place in zebrafish (Takahashi 1977), strongly suggests the use of labile life-stages in the assessment of EDC-exposed reproductive pressures in fish.

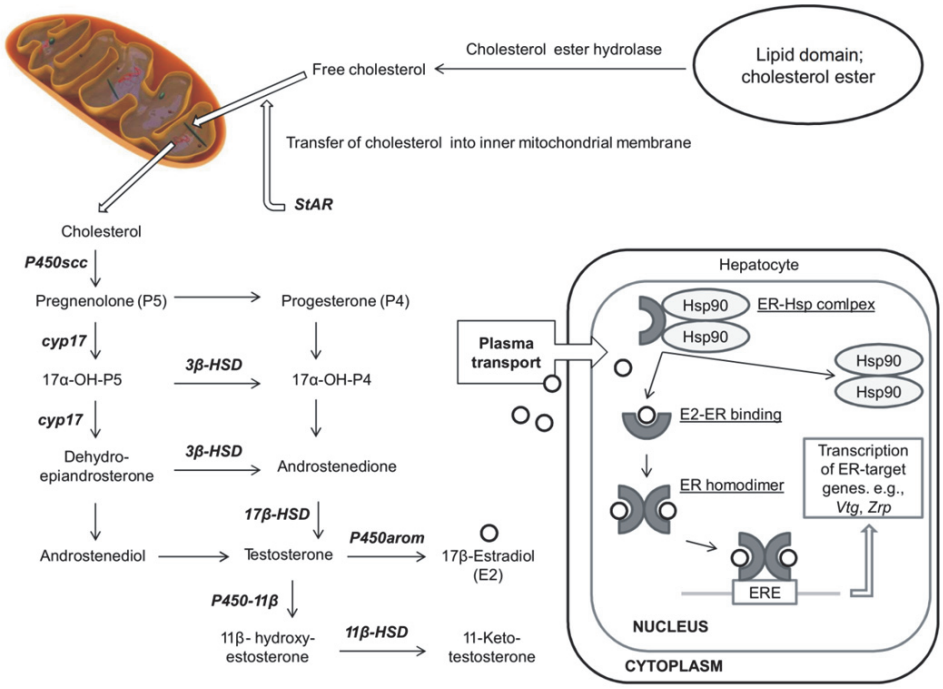


FIGURE 1 Generalized representation of the gonadal steroidogenic pathways and the action of estrogen on transcription of estrogen-responsive genes (*vtg*, *zrp*) in fishes. Under the influence of pituitary gonadotropins, E2 is produced in the ovarian follicle cells. P450scc, cytochrome P450 side-chain cleavage; Cyp17, cytochrome P450 17α-hydroxylase/17, 20-lyase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; ERE, estrogen receptor element; Vtg, vitellogenin; Zrp, zona radiata protein; Hsp90, heat shock protein 90. Depicted according to several sources (Baroiller *et al.* 1999, Carvan *et al.* 2005, Hiramatsu *et al.* 2005, Thomas 2008).

Nuclear receptors are a superfamily of transcription factors that are activated by binding ligands, both endogenous and exogenous, and mediate signaling over various gene networks (Hiramatsu *et al.* 2005). Mode-of-action (MoA) of xenohormonal compounds that mimic endogenous steroids are mediated by binding to steroid receptors, a subfamily of nuclear receptors. While commonly identified effects of EDCs on fish physiology include impact on estrogen receptors (ERs), they also mediate signaling of relevant gene-level responses downstream of the steroid-regulatory pathways (Fig. 1). Regulatory actions of ERs are broadly categorized into genomic and non-genomic pathways, depending on the specificity of the ligand (Heldring *et al.* 2007, Shanle and Xu

2011). The genomic signaling pathway, shown in Fig. 1, includes ER-ligand interaction within the cell and the direct DNA binding at EREs to mediate target gene regulation. Non-genomic ER signaling includes ligands bound to membrane-associated receptors, which cause activation of kinase signaling cascades resulting in rapid physiological responses without gene regulation. Heldring *et al.* (2007) also discussed the MoA of ligand-independent pathways, which involve activation by the growth factor signaling pathway, causing phosphorylation of ERs, dimerization, and DNA binding leading to gene regulation.

1.2.2 ER- and AhR-mediated "cross talk" in endocrine responses

Formation of ligand-receptor complexes is central also to other key biological pathways, such as xenobiotic metabolism with aryl hydrocarbon receptor (AhR), the responsible receptor (Fig. 2a). The AhR may interact (cross-talk) and inhibit ER-signaling by possible mechanisms, including enhanced metabolism of E2, inhibition of ER-mediated gene transcription, co-regulation of ER-dependent transcription, proteasomic degradation of ER or competition (Safe and Wormke 2003, Matthews and Gustaffson 2006), to modulate transcription of ER and associated genes (Bemanian *et al.* 2004, Mortensen and Arukwe 2007) (Fig. 2b).

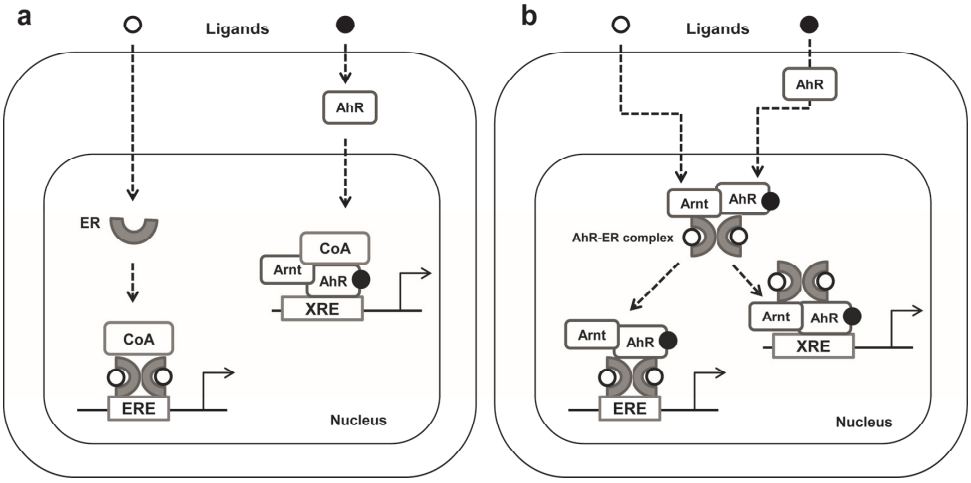


FIGURE 2 Schematic representation showing (a) typical ligand-dependent regulation of ER- and AhR-mediated genes, and (b) crosstalk pathway with interaction between ER- and AhR-signaling in a vertebrate animal. AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; CoA, coactivator, ER, estrogen recptor; ERE, estrogen receptor element; XRE, xenobiotic response element. Note that open- and closed-circles represent ER-agonist and AhR-agonist, respectively. Modified from Ohtake *et al.* (2011).

Therefore steroid receptors, such as ER, may not alone adequately explain the reproductive outcome in fish exposed to endocrine-active ligands without

interaction with other signalling pathways (e.g. AhR). For instance, many exogenous ligands as substrates for xenobiotic biotransformation, as well as being central to activation of estrogen-responsive genes, should be considered. The induction of *cyp1a*, a biomarker of xenobiotic biotransformation mediated by AhR, is known to show negative correlation with estrogenic response in teleosts (Arukwe *et al.* 2008, Gräns *et al.* 2010).

Toxicity studies using *in vivo* models show authentic responses, since they are based on the acclimatory physiology of the animal; specific to the immediate exposure scenario. Increasing focus has been recently directed to natural and synthetic chemicals known to alter reproductive health of human and wildlife through modulation of physiological estrogenic and androgenic functions. By definition, estrogenicity *per se* is not an adverse effect, as focused, e.g., in females, although disruptions along related pathways often amount to altered homeostasis and regulation of the endocrine system. Compared to the roles of CYP1A and CYP3A in mammalian species, the metabolism of E2 in zebrafish is mainly catalysed by CYP1A and CYP1C enzymes with the formation of 2-hydroxyestradiol (2-OH E2) being the dominant metabolite (Scornaienchi *et al.* 2010). Depending on ligand-activation, both ER and AhR are known to play inhibiting and co-regulating roles in signalling of either pathway (Hasselberg *et al.* 2005, Matthews and Gustaffson 2006, Gräns *et al.* 2010, Ohtake *et al.* 2011, Bugel *et al.* 2013). Specific to oviparous teleosts, anti-estrogenic effects such as decreased vitellogenesis can be mediated also by AhR, activated by AhR agonist contaminants (Gräns *et al.* 2010, Bugel *et al.* 2013). Being ligand-activated transcription factors, ERs and AhRs and their interactions are critical to the net outcome of subsequent biological events manifesting in synthesis and metabolism of key factors, i.e. sex steroids.

1.2.3 Exposure to chemicals as mixture

Risk assessment of surface waters has become increasingly challenging owing to the higher volumes of pharmaceutical, domestic, industrial and commercial discharges, which generate complex mixtures with numerous combinations and unknown net toxicities (OSPAR 2007). The potential effects of low-level concentrations of chemicals acting on long-term endpoints, like growth and reproduction, appear to redefine the concept of environmental dose. This is due to biological effects with multiple components in an environmental mixture that may act unexpectedly regarding their combined capacity of impacts (Sumpter and Jobling 1995). Overall, estrogenic chemicals in mixtures may show a combination of effects in aquatic models (Thorpe *et al.* 2001, Brian *et al.* 2005, 2012, Lin and Janz 2006, Sun *et al.* 2009, Zhang *et al.* 2010, Petersen and Tollefsen 2012).

Evidence for adverse reproductive responses in fishes exposed to municipal sewages and kraft mill discharges constitute considerable proof that most realistic environmental contamination is a product of combination, and not of isolated chemicals (Beyer *et al.* 2014). The prediction of non-interactive joint effects is based on the concentration addition (CA) model, where mixture-

components with similar MoA are considered, whereas the independent action (IA) model, also named as response addition, predicts combined response based on different modes of action (Hertzberg and MacDonell 2002). Few *in vivo* sublethal assays with fish species have documented the applicability of the CA model in estimating combination effects (Zhang *et al.* 2010, Brian *et al.* 2012). In particular, the deviations from additive responses, called synergism or antagonism, may result from interactive effects of components in a mixture (Fig. 3). Xenoestrogenic chemicals act additively at environmentally relevant concentrations (Brian *et al.* 2005), though synergism in laboratory studies is not common (*in vitro*, Silva *et al.* 2002, Staal *et al.* 2007; *in vivo*, Sun *et al.* 2009). Somewhat surprisingly, current regulatory emphasis in Europe, as enacted by European chemical legislation such as REACH and the Water Framework Directive (WFD), discuss toxicity of chemicals with similar MoA (Syberg *et al.* 2009, SCHER, SCCS, SCENIHR 2012, Beyer *et al.* 2014). The practise underestimates the potencies and roles of sub-threshold concentrations of chemicals with dissimilar MoA occurring as environmental mixtures. In reality, exposure scenarios are complicated when environmental mixtures include contrasting MoA such as, estrogenic and anti-androgenic components.

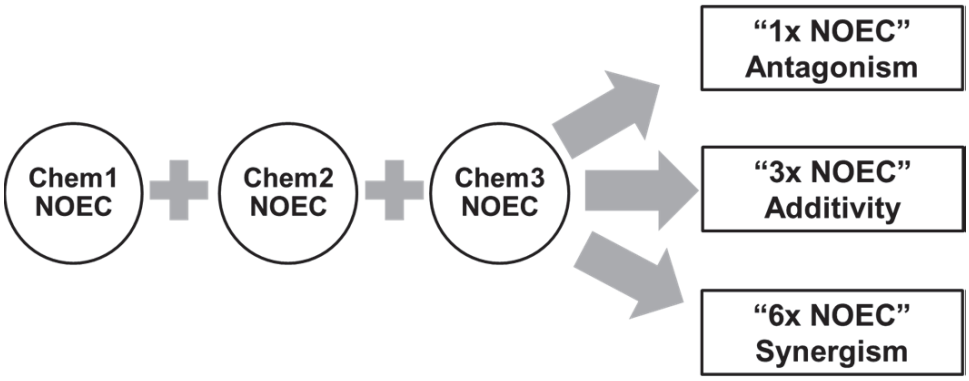


FIGURE 3 Schematic of combined effects including non-interactive (additive) and interactive (antagonistic and synergistic) scenarios of individual components in a mixture.

1.2.4 Exposure to sediment-bound contamination

Hydrophobic organic molecules tend to sorb onto organic moieties in natural waters and sediments, often reversibly (Schwarzenbach *et al.* 2003). In an aquatic system, given the linear relationship between log K_{ow} and the organic carbon-water partition coefficient (log K_{oc}), ionic constituents of the water column control the partitioning chemicals into sediment, thereby influencing bioavailability (Erickson *et al.* 2008). Uptake of a chemical by fish either from sediment or, once desorbed, from a freely dissolved fraction in the aqueous

phase, depends on the sorption capacity of the chemical described by the solid-water partition coefficient K_d (L kg^{-1}).

$$K_d = C_s / C_w = K_{oc} \times f_{oc}$$

where C_s and C_w are the concentrations of the chemical bound to solid (mol kg^{-1}) and dissolved in aqueous phase (mol l^{-1}), respectively, at equilibrium. K_{oc} is the solid-water partition coefficient normalized for the organic carbon content for the solid (L kg^{-1}), and f_{oc} the weight fraction of the organic carbon in the solid phase (kg kg^{-1}). Dissolved organic matter (DOM) and colloids may significantly influence the transport and fate of chemicals in the aquatic systems, leading to accumulation in the solid or sediment phase. The octanol-water partitioning coefficient, K_{ow}, of the chemicals with estrogenic impact effectively predicts the partitioning behaviour based on their hydrophobic nature ($K_{ow} \geq 3$). The K_{ow} value also predicts the competitive binding of sites available in the sorbent phase (Erickson *et al.* 2008). Another major factor contributing to the environmental fate and persistence of estrogenic chemicals is the minimal or even lack of biodegradation in the anaerobic sediments (Ball *et al.* 1989). These factors point to the relevance of investigating multiple exposure scenarios, for instance active foraging in sediment by animals, rather than limiting investigation to water-borne exposures only.

1.3 Zebrafish as screening model of xenoestrogenicity

1.3.1 General information

The zebrafish, *Danio rerio* (Hamilton 1822) has been widely used as a vertebrate aquatic model owing to its practical advantages, including cost-effective maintenance, asynchronous breeding, and rapid development over the embryo-larval juvenile stages. Among other small-bodied species, such as the fathead minnow *Pimephales promelas* (Panter *et al.* 2000, Johns *et al.* 2011) and Japanese medaka *Oryzias latipes* (Melo and Ramsdell 2001, Han *et al.* 2010), it is routinely used for basic and applied endocrine studies of chemicals used in the environment and environmental samples. With the flexibility of assessment endpoints, life-stages of zebrafish from post-fertilized egg through embryonic, juvenile up to adult animals have been widely used to study developmental (Kimmel *et al.* 1995), mutational (Incardona *et al.* 2004), teratogenic (Brannen *et al.* 2010) and reproductive (Andersen *et al.* 2003, Van den Belt *et al.* 2003, Segner *et al.* 2003) impacts among other toxicity investigations. Specifically, endocrine status has also been widely studied in zebrafish ELS (Kishida *et al.* 2001, Andersen *et al.* 2003, Brion *et al.* 2004, Kazeto *et al.* 2004, Muncke and Eggen 2006).

Progress in existing *in vivo* screening of vertebrate gene function has seen the increased use of transgenic and morpholino antisense oligos or gene

knockdown technologies (Carvan *et al.* 2005, Hill *et al.* 2005, Hahn and Hestermann 2008), and in particular the induction of green fluorescent protein expression in transgenic zebrafish, *cyp19a1b-GFP* (Tong *et al.* 2009, Brion *et al.* 2012) and *ere-zvtg1: gfp* (Chen *et al.* 2010), presenting non-lethal alternatives in animal testing. Furthermore, the zebrafish as a model is a well investigated species with large genetic and genomic resource databases e.g. Zebrafish International Resource Center (ZFIN, Bradford *et al.* 2011), including several wild strains.

1.3.2 Relevancy of early life-stage effects in environmental risk assessment

Anamniotic eggs of fishes and amphibians develop in surface waters and present unique scenarios of chemical exposure. Sexual differentiation in the teleostean brain determines the fate of the gonads (Fig. 4), contrasting with amphibians, often targeted in toxicological studies (Francis 1992).

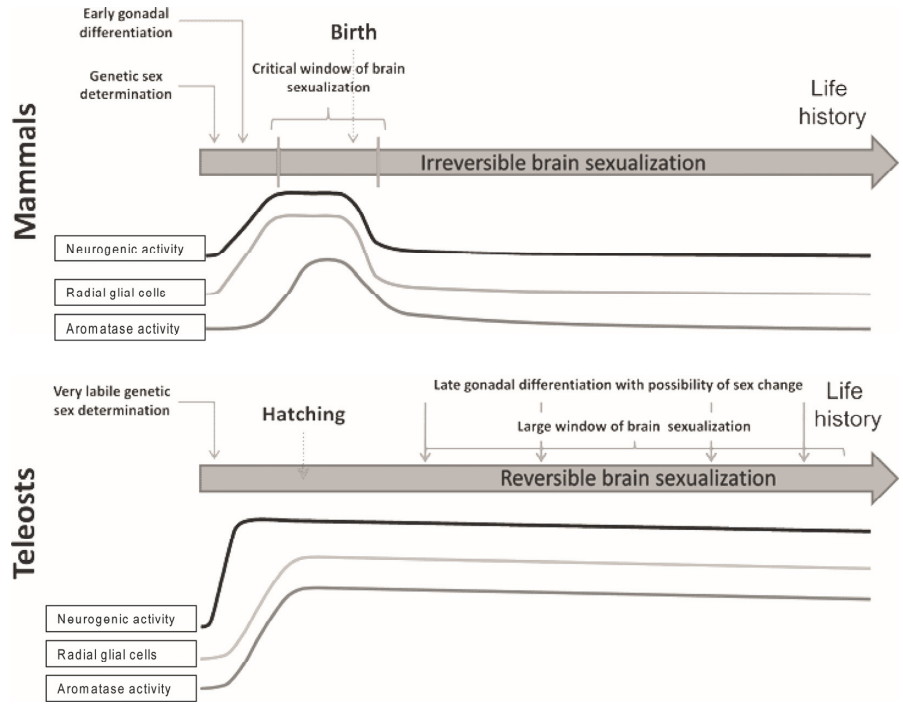


FIGURE 4 Schematic representation of the life history in fish compared to mammals, highlighting the major differences that may affect the process of brain plasticity (From Le Page *et al.* 2010, reproduced with permission).

In gonochoristic species, sex-specific mature individuals possess either ovaries or testes. Gonochoristic teleosts are further categorized into sexually undifferentiated species such as zebrafish that initially possess ovary-type gonads, which later develop into functional male or female gonadal tissues. In

contrast, during ontogenesis, sexually differentiated species that differentiate directly into a testis or ovary (Piferrer 2001) are less sensitive to the impact of steroid exposure than undifferentiated species (Yamamoto 1969). In presumptive males, the juvenile ovary stage subsequently develops into a testis by possible apoptotic loss of immature oocytes constituting the immature ovary (Uchida *et al.* 2002). Although all mechanisms for phenotypic sex-determination are not yet fully understood in zebrafish, an initially female development of protogynous fish that show hermaphroditism with a female-to-male sex change presents suitable candidate species for finding chemical or environmental influences on gonadal and phenotypic plasticity during ontogenesis. Piferrer (2001) defined the sexually labile period as "… the process taking place at the undifferentiated gonad that determines the future course of sex differentiation towards a particular phenotype."

Unlike mammalian vertebrates, teleost fish have a large time window of gonadal differentiation, which significantly influences the final sexualisation (masculinization or feminization), so that an individual is susceptible to social, environmental and (xeno-) hormonal signals. Teleosts are therefore ideal ecotoxicological models since they exhibit both genetic as well as environmental modes of sex determination. Sex-reversal of female zebrafish exposed to temperature or steroidogenic modulators (Uchida *et al.* 2002, 2004) strongly supports the delayed brain sexualisation hypothesis (Piferrer 2001, Le Page *et al.* 2010) (Fig. 4). Sex-reversal in mature male medaka corresponded with dose-dependent exposure to synthetic E2 (Melo and Ramsdell 2001), and also with other chemicals (Paul-Prasanth *et al.* 2011) and emissions (Edmunds *et al.* 2000).

1.3.3 Choice of a zebrafish life-stage for estrogenic evaluation

Age-specific developmental factors, including chorion-permeability and the yolk sac, of embryo and hatched eleutheroembryo-stages are frequently considered when selecting a suitable early life-stage for *in vivo* assays. Both these life-stages present limiting or confounding scenarios for chemical exposure. First, fertilised eggs contain maternally invested yolk which formed during vitellogenesis in the adult female. Yolk, besides being the energy and maternal source until the free-feeding stage of the developing fry, is also the route of exposure to lipophilic toxicants transferred from the mother fish. Secondly, whereas the presence of chorion encapsulating the embryo is a barrier to unrestricted chemical exposure (Lillicrap 2010, Henn and Braubeck 2011), the yolk sac of eleutheroembryos acts as a sink for lipophilic chemicals (Andersen *et al.* 2003). For instance, structural composition of the zebrafish embryo at the gastrula stage (7 hpf) includes a three-layered chorion enveloping yolk at the core surrounded by a syncytial layer and perivitelline space (Rawson *et al.* 2000). Further, the chorion membrane complex consists of three layers, with pore canals permeating the middle and inner layers. These channels are suggested to be the route of solute transfer and permeability, which are further influenced by lipophilicity (Wiegand *et al.* 2000, Lammer *et al.* 2009), molecular size (Henn and Braunbeck 2011), ionic interaction on the

chorion surface (Pullela *et al.* 2006) and the xenobiotic efflux transporter (Fischer *et al.* 2013), besides being dependent too on environmental factors. Although all mechanisms of chorion permeability are not fully understood, the most compelling approach is based on the ionic composition of the chorion surface; acting as ion exchanger or barrier depending on the binding molecule. Additionally, chorion permeability to industrial wastewater changes according to the developmental life-stage (Gellert and Heinrichsdorff 2001), which generally interferes with a steady and specifically controllable exposure regime.

For direct exposure to ambient factors by an embryo, a practice of dechorionation by physical means (Westerfield 2007, Henn and Braunbeck 2011) as well as by automated techniques (Mandrell *et al.* 2012) have been introduced. Technically, dechorionated embryos (24 hpf) and eleutheroembryos showed similar toxicity response to exposure by Luviquat, a catioinic polymer (Henn and Braunbeck 2011), though being labour-intensive may not be practical for routine toxicity testing. Further, no dechorionation takes place in nature, which implies that this is an atypical route to elicit toxic response in the embryo, even if it may hold relevance in basic developmental biology.

Thus the restricted uptake and elimination of maternally transferred pollutants in a fertilized fish egg and in early embryos, compared to post-hatch fry (Foekema *et al.* 2012), is associated with the chorion acting as a more or less impermeable barrier to ambient xenobiotics and with the relatively small surface–volume ratio of an egg in comparison to that of a hatched fry (Petersen and Kristensen 1998). Expression of estrogen-responsive genes, including vitellogenin isoform *vtg1*, have been shown to have identical sensitivity in embryonic (exposed over 0–3 dpf) and juvenile (17–24 dpf) zebrafish exposed to E2, a typical lipophilic chemical (log K_{ow} 3.94, Lai *et al.* 2000) at LOEC, 0.25 µg l^{-1} (Jin *et al.* 2009). Within the context of exposure reaching the embryo across the chorion, there was clear dose-dependence shown by Jin *et al.* (2009). On the other hand, use of a pre-hatch life-stage in sediment toxicity exposures can be limited in terms of including exposure from sources (routes) other than the ambient water medium.

For our studies, the free-swimming and free-feeding life-stage of the 20 days-post-fertilization zebrafish (*20dpfZF*) was therefore chosen to actively forage food items from water as well as from around the sediment surface in the exposure chambers. Thus *20dpfZF* accommodates the possibility of different routes of exposure: water-borne and chemical adhered-to food and sorbed to sediment particles, i.e. trophic transfer. The selection of the life stage also considered the period post-hatch, where any mortality in the cultured batch had stabilized, when the hatched fry after depleting the yolk was at a free-feeding life-stage. Furthermore, juvenile zebrafish undergo a labile phase (15 to 42 days post fertilization; dpf) of gonadogenesis (Takahashi 1977, Maack and Segner 2004) (Fig. 5). An ecological fact is that a developing juvenile needs to feed actively to support its growth and development, so an ecotoxicological assay is required to accomodate this key event to allow for proper extrapolation towards the population metrics. Additionally, because in general exogenous or

synthetic steroids may elicit more pronounced sex-reversal in fish than natural steroids do (Piferrer 2001), this phenomenon, coupled with the reduction in use of adult animals in toxicity studies, presents a unique probing model in the early juvenile zebrafish.

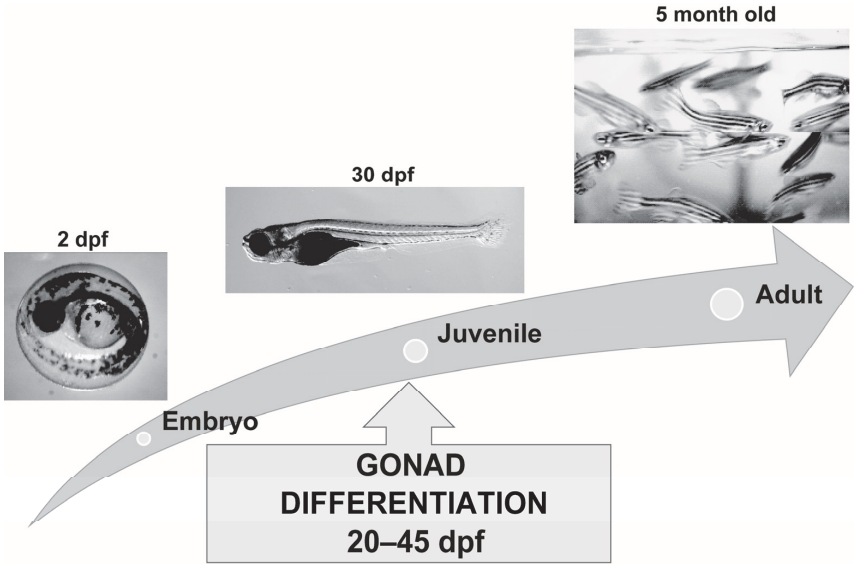


FIGURE 5 Life-stages of zebrafish (wild-type). Free-swimming juvenile zebrafish (20-25 dpf) represent the labile life-stage responsive to endocrine modulation. dpf = days-post-fertilization.

1.4 Biomarkers of endocrine modulation

Following evolutionary divergence 350 million years ago, teleost fishes have retained ca. 15-30 % of the duplicated genes, including cytochrome P450 aromatase (Cyp19), ERs and aryl hydrocarbon receptors (AhRs) among others (Hahn and Hestermann 2008). The CYP, or cytochrome P450 system, plays a key role in the metabolism of the exogenous and endogenous compounds. Of particular significance to steroid biosynthesis and metabolism is Cyp19, as its expression and activity is under the control of hormones and hormonal mimics. For instance, sexual dimorphism in male medaka was shown to be dependent on E2 exposure driven by brain aromatase activity (Melo and Ramsdell 2001). Although Cyp19 has been associated also with zebrafish sex determination (von Hofsten and Olsson 2005), duplicated genes of Cyp19 have been shown to be tissue-specific (gonad, *cyp19a1a*; brain, *cyp19a1b*; Callard and Tchoudakova 1997, Callard *et al.* 2001, Chiang *et al.* 2001). This fact, along with the significantly higher relative expression of the brain isoform than that in gonads, has been widely used to establish estrogenic stimulation of sex steroid synthesis (Callard *et al.* 2001, Kishida *et al.* 2001). The presence of ERE in the promoter

region of neural aromatase gene (*cyp19a1b*), but its absence on the gonadal isoform (*cyp19a1a*), (Callard *et al.* 2001, Kazeto *et al.* 2001, Diotel *et al.* 2010), have been shown to explain the estrogen-responsiveness of *cyp19a1b* in embryonic zebrafish between 24-48 hpf (Kishida and Callard 2001). Such a robust expression of *cyp19a1b* in embryonic zebrafish highlights the neuroendocrine control of fish reproduction, and mostly in relevant developmental time windows that are central to sex-differentiation in zebrafish and other undifferentiated gonochoristic fish species.

In oviparous vertebrates, Vtg synthesis takes place in the liver and is strongly controlled by circulating E2. The presence of Vtg, normally occurring in females, also in male fish, is known to be negatively correlated with testicular growth and maturation in adult fish (Jobling *et al.* 1996). Classification of multiple teleost Vtgs (types A, B and C) was based on the composition of their protein domain structure (Hiramatsu *et al.* 2002, 2005). Furthermore, the diversity of Vtg regulation in zebrafish alone, with at least seven Vtg genes, show age-, sex-, and tissue-dependent expressions, with *vtg1* being the most pronouncedly expressed in the adult (Wang *et al.* 2005) as well as in embryonic zebrafish, as early as 48 hpf when exposed to EE2 (Muncke and Eggen 2006). The expression of *vtg* in E2-treated adult zebrafish, female (intestine and ovary) and male (intestine and muscle) was also shown to be extra-hepatic (Wang *et al.* 2005). Subsequent responses include the physiological production of proteins (Vtg) over long-term exposures. The half-life of vitellogenin protein, being 2.4 d in juvenile zebrafish exposed from 0–25 dph to 15 ng EE2 l^{-1} (Andersen *et al.* 2003), is an important information, along with higher stability of Vtg mRNA relative to the protein level (Muncke *et al.* 2007). However, vitellogenin protein half-lives in some cold water species, such as flounder (15 d) and plaice (35 d), were significantly longer than the respective half-lives of mRNA, at 2.8 and 3.1 days respectively (Craft *et al.* 2004). The feasibility of detecting altered gene response even at low sublethal concentrations has been suggested as a reliable indication for short-term *in vivo* exposures (Scholz *et al.* 2004). Though elevated plasma Vtg (protein) in adult fish may indicate pollutant-induced vitellogenesis, it could also result from the natural and breeding-related alterations along the HPGL axis. The results of the studies included in this thesis are indicative of such changes at the gene-level.

2 OBJECTIVES

The primary focus of this thesis was to identify and evaluate the ecotoxicological relevance and sensitivity of an early life-stage (ELS; 20dpf) of zebrafish *Danio rerio* to model estrogenic chemicals and heterogenously polluted sediments, with emphasis on gene-level responses to xenoestrogen-induced effects *in vivo*. Key gene biomarkers were identified to indicate steroidogenic alteration along the HPGL axis, overlapping with the gonadal differentation of the *20dpfZF*. The utility of the *20dpfZF* was explored as a screening model for environmental samples with endocrine modulating potential.

The objectives that were explored in this thesis included:

- *In vivo* zebrafish responses to common xenoestrogens, bisphenol A (BPA), ethinylestradiol (EE2), nonylphenol (NP) and octylphenol (OP), extending the sensitivity to sediment-borne estrogenic (EE2) exposure (I)
- Effects of fixed-ratio mixtures of xenoestrogens on estrogen-responsive gene transcripts (II)
- Sensitivity of the *20dpfZF* exposed to sediment spiked with an androgenic chemical, tributyltin (TBT), and the applicability of the *in vivo* model in assessing the endocrine modulation by a TBT-contaminated industrial sediment (III)
- Probing of endocrine effects of a sediment site contaminated by pulp and paper processing activities (IV)

3 MATERIALS AND METHODS

3.1 Chemicals

The chemicals studied were bisphenol A (BPA, purity 99+ %), 17α-ethynylestradiol (EE2**,** minimum 98 % by HPLC), tributyltin chloride (TBT-Cl, minimum purity 96 %) and 4-*tert*-octylphenol (OP, 97 %) and purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-n-Nonylphenol (NP, 98+ %) was obtained from Alfa Aesar **(**Karlsruhe, Germany). Silylation reagent *N*, *O*-bis (trimethylsilyl) trifluoro acetamide (BSTFA) with 1 % trimethyl chlorosilane (TMCS) was purchased from Fluka Chemie (*Buchs*, Switzerland). Internal standards 17-α-ethynylestradiol-d4 (EE2-d4, 97–98 %), and 2, 2-bis (4-hydroxyphenyl) propane-d16 (BPA-d16, 98 %) were from LGC Standards AB (Borås, Sweden). The solvents, methanol (MeOH) and acetonitrile, supplied by Merck Chemicals (Darmstadt, Germany) were of HPLC grade. Separate chemicals were dissolved and diluted in MeOH, and stocks were stored at –20 °C in the dark until use, which was within two weeks.

3.2 Zebrafish husbandry and breeding

Adult male and female zebrafish (*Danio rerio*) (wild type; WT) were obtained in 2011 from the Institute of Medical Technology, University of Tampere (Tampere, Finland). Fish were acclimatized to temperatures (mean ± SD, 26 ± 1 °C) and to a photoperiod (14 h light/10 h dark) for at least two weeks prior to setup for breeding (Fig. 6). Animal husbandry was carried out along established guidelines (Westerfield 2007). Zebrafish adults were fed twice daily, the food alternating between dry granules (SDS diets, UK), frozen chironomid larvae (Ruto frozen fish food, Zevenhuizen, Holland) and freeze-dried tubifex (JBL NovoFex, Neuhofen, Germany). Following breeding between sexually mature adults (~8 month old), fertilized eggs (+2 hours-post-fertilization; hpf) were

collected and reared through early post-embryonic stages for 20 days (*20dpfZF*) in rearing water or embryo medium (Westerfield 2007). Post-hatch zebrafish from 6 dpf through 20 dpf were fed with fry food (SDS diets, UK), and depended on the sufficient survival (>90 %) of reared fish by 10 dpf, whereafter the experimental animals were selected at 20 dpf. Experiments were conducted according to and licensed by the Finnish authority for animal experiments (ESAVI-2010-07885/Ym-23).

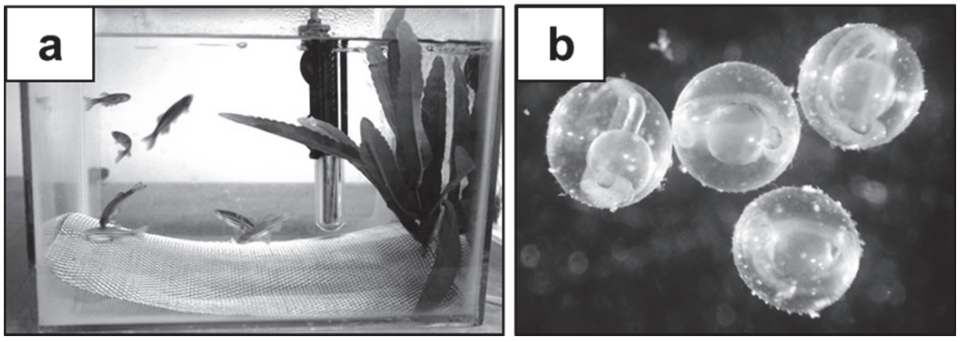


FIGURE 6 Breeding setup for adult zebrafish. (a) Breeding pairs (4 ♂ + 3 ♀) are placed in 10 l water system with mesh or marbles at the bottom of the tank. Water temperature is set at 28 °C overnight, and (b) fertilized eggs (+ 2 hpf) are collected from under mesh or between marbles on the following day.

3.3 Study areas and sediments

The study areas located in Central and Southeastern Finland are shown in Fig. 7 (III, IV). The OTC-contaminated sediment (ca. 0–10 cm depth) was collected from Lake Huruslahti (Varkaus, Central Finland) (Fig. 7a) (III). The target sampling location was a highly polluted harbour and chemical wood industry area (Anon 2009). Other chemical contaminants in the area also include residues from variable industrial processes and products from the past, including chromium, copper, mercury, nickel, and zinc that were discharged in the bay. Clean reference sediment (0–10 cm depth) was sampled from Lake Palosjärvi (Toivakka, Central Finland). The second study area was located near the city of Lappeenranta, in the watercourse downstream from the Kaukas Inc. pulp and paper mill (PPM), that flows into Southern Lake Saimaa (SLS), a large oligotrophic lake in SE Finland (Fig. 7b) (IV). Lake Rautaniemi (RN), 9 km upstream from the mill, was considered to be a reference site with waters and sediments not directly impacted by the mill discharges. The two downstream sites (1 and 3 km), were selected as polluted sites suitable for studying the impact of PPM-contaminated sediment. The sediment quality of the SLS study area has been reported in earlier studies (Karels *et al.* 2000, Lahdelma and Oikari 2006, Ratia *et al.* 2014).

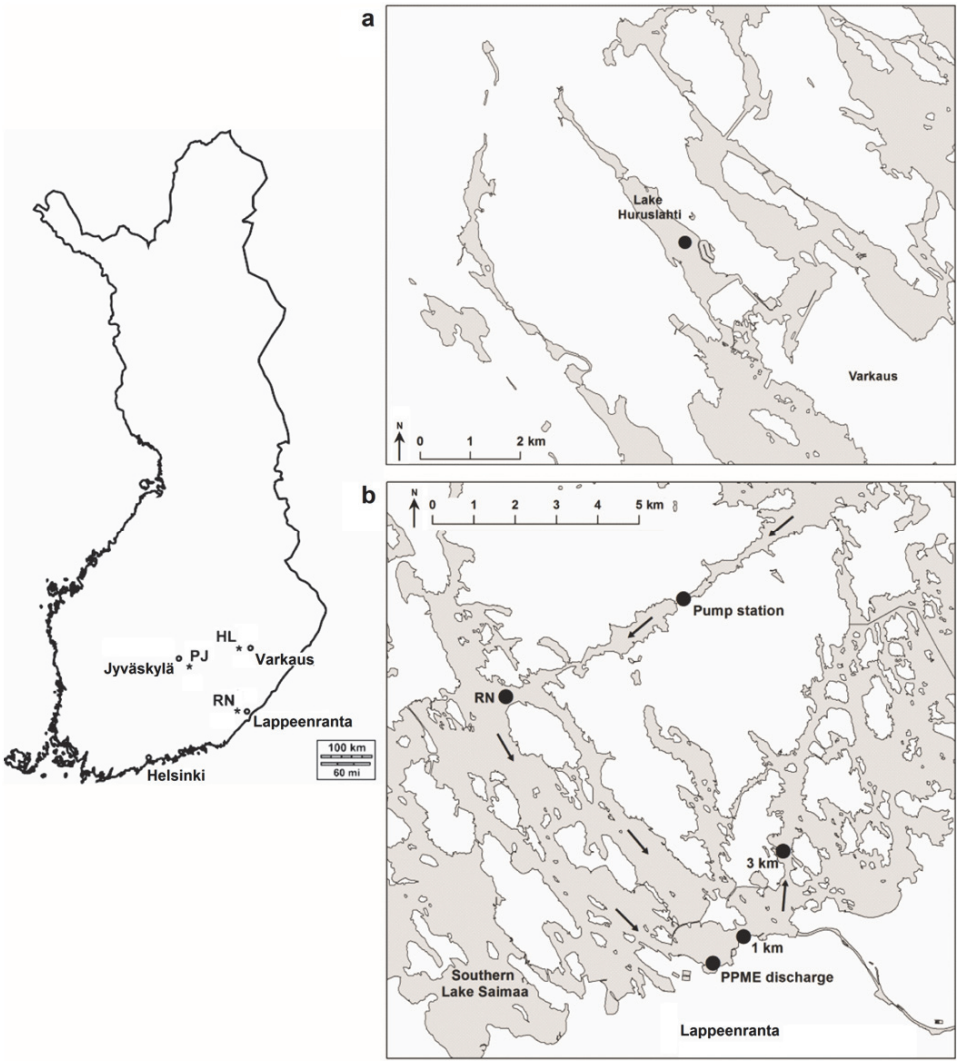


FIGURE 7 The study areas in Central and Southeast Finland (III, IV). Sampling sites of reference (PJ, Lake Palosjärvi; RN, Lake Rautniemi) and contaminated sediment from (a) Lake Huruslahti (HL) in Varkaus and (b) 1 km and 3 km downstream sites from mill in southern Lake Saimaa (SLS) located in Lappeenranta. Sites for sample collection are represented by closed circles. Map data © National Land Survey of Finland, 2013.

3.3.1 Sampling of sediments

The OTC-contaminated sediment (0–ca.10 cm depth) used in Study III was collected from Lake Huruslahti (Varkaus, Central Finland), with the aid of an Ekman-Birge type bottom sampler, in October 2011. About 30 lots were combined, representing an area of several acres. Clean reference sediment was similarly sampled from Lake Palosjärvi (Toivakka, Central Finland) and,

comprised ca. 20 lots. Combined matter was homogenised to one study sample, and transported to the laboratory and stored in the dark under ca. 10 mm water layer at 4 °C prior to further analysis and processing. The sediment samples are designated PJ (Palosjärvi) and HL (Huruslahti). In Study IV, sediment samples from reference as well as downstream polluted sites were collected in May 2011. Five sediment cores (0–5 and 20–30 cm depth) were collected from the two downstream sites using a Limnos sediment sampler (Fig. 8a), and a slicer (ca. 1 cm accuracy) was used to combine samples to form upper and lower core depths (Fig. 8b). Clean reference sediment was sampled from RN, using an Ekman-Birge sampler. Combined sediment cores, 0–5 and 20–30 cm sections, represent recent and historically polluted sediment, respectively. The wet sediment samples were stored in the dark at 4 °C under anaerobic conditions until further analysis and use. Dry weight of samples was determined according to Nikkilä *et al.* (2001).

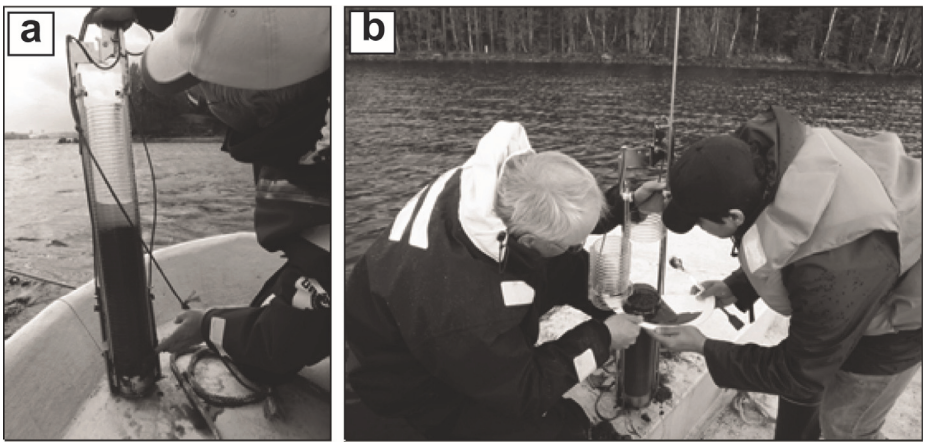


FIGURE 8 Limnos corer (a) used to sample ca. 0–30 cm sediment sections. A slicer (b) is used to collect the recent (ca. >2005) and historical (ca. < 1992) samples of 0–5 and 20–30 cm depths, respectively. Photo courtesy of Aarno Karels.

3.3.2 Preparation of sediments for whole sediment bioassays (I, III, IV)

To harmonise a similar organic matter for all sediment assays of EE2 and OTC (I, III, IV), inorganic constituents were added when needed for dilution. Specifically, the native reference sediment (PJ) was reconstituted with inorganic constituents (51 % fine-grained sand, 0.05–0.25 mm; 26 % medium-coarse sand, 0.3–0.6 mm; 17 % kaolin, Riedel-de Haën, Germany), with minor modifications to the method employed by Nikkilä *et al.* (2001) (I, III, IV). The reconstituted sediment was slowly stirred using a stainless steel blender at 4 °C for ca. 8 h. Following the mixing, the reconstituted reference sediment (PJ) was aged for ca. 8–10 months before use in bioassays (I, III, IV), including preliminary trials. To represent sediment with estrogenic potential, reference sediment PJ, adjusted to

similar TOC (%) as RN was spiked with 3 µg EE2 kg^{-1} dw, and allowed to equilibrate (> 6 months) before use.

Spiking of the reference sediment (PJ; reconstituted Palosjärvi with TOC 4.3 % dw) with TBT-Cl was performed according to Burton *et al.* (2006). Briefly, nominal concentration 10 mg TBT kg^{-1} (dw) was prepared by gradually administering TBT (dissolved in 100 % methanol) to sediment slurry with a minimum amount of water and mixed for ca. 7 h. Following the addition, the headspace of the sample container was filled with an inert gas (N_2) and sealed for equilibration at 4 °C for over 60 d (ASTM 2000). The spiked-sediment, PJ + TBT (10 mg kg^{-1} dw; equivalent to 100 % TBT-spike) was subsequently mixed with PJ at 4 °C for 8 h, to constitute a nominal dilution series of 10, 33 and 100 % TBT contamination. Similarly, mixing ratios of HL + PJ resulted in 10, 33 and 100 % HL. The diluted sediments were further aged under anaerobic conditions at 4 °C for ca. 6–8 months before experimental use.

3.4 Laboratory exposures

3.4.1 Water-borne exposure of xenoestrogens with *20dpfZF* (I)

Chemical exposures were conducted in 400 ml glass jars containing 250 ml of gently aerated embryo-rearing water (pH 7.2) for the duration of the experiment. Water-borne exposures (I and II) were carried out in a semi-static manner, replacing 100 % of the medium daily to minimize change in water quality. Water quality parameters were measured on days 1 and 4. The mean temperature in the exposure jars was in the range 24–26 °C. The content of dissolved oxygen (DO, mg l^{-1}) was 7.6 ± 0.2 (mean DO ± SD) and pH 7.2 ± 0.2 (mean ± SD). Chemicals dissolved in the carrier solvent, methanol (MeOH) were administered to the exposure water, the solvent concentration being 0.02 % or less. Preliminary exposures also included pure water controls (data not shown), i.e. without solvent to confirm similarity with that of the maximum solvent concentration (0.02 %).

The first set of exposures (Exposure I) were performed with groups of 30 fish (*20dpfZF*), replicated three times in parallel, exposed to 0.02 % MeOH with 0, 5, 25 and 50 ng EE2 l^{-1}, to determine the expression and selection of suitable steroidogenic gene(s). The second set of exposures (Exposure II) were performed with groups of 25 fish (*20dpfZF*), replicated three times, exposed to 0.02 % MeOH with 0, 5, 25, and 50 ng EE2 l^{-1}, 0, 100, 500, and 1000 µg BPA l^{-1}, 0, 10, 50, and 100 µg NP l^{-1}, and 0, 10, 50, and 100 µg OP l^{-1}. Although the results used in the discussion represent measurement from three parallel replicates, the patterns of relative expression of target genes were also verified with two independent exposures conducted earlier. Fish were sampled at the end of five days of exposure. Each group of sampled animals (n = 15) was collectively weighed in RNase-free micro centrifuge tubes (Starlab, Germany), and snap-frozen in liquid nitrogen and stored at –80 °C until further analysis.

3.4.2 Water-borne exposure of BPA, NP and OP as mixture (II)

Chemical exposures were also semi-statically conducted in glass jars containing 250 ml of exposure medium, consisting of embryo-rearing water (pH 7.2) for the duration of the experiment. The level of water quality, including temperature (25 ± 1 °C mean ± SD), dissolved oxygen (7.6 ± 0.2 mg l^{-1}) and pH (7.2 ± 0.2), was maintained for the entire exposure period. Each triplicate exposure jar contained 25 fish (*20dpfZF*) with 100 % water renewed daily throughout the five-day exposure. All exposure solutions were prepared using MeOH (0.01 %) as a solvent carrier. First a pilot study was performed, in which *20dpfZF* were exposed to nominal concentrations of BPA at 125, 250, 500, 1000 and 2000 µg l^{-1}; NP at 10, 50, 100 and 200 µg l^{-1}; and OP at 5, 10, 50 and 100 µg l^{-1}, to determine the no-effect threshold concentration (NOEC) of each chemical for estrogenic gene induction. Based on these results (I), the concentrations for the main studies with the fixed combinations were selected (II). As fixed combinations, the highest single concentrations with statistically non-significant gene induction, i.e. at NOECs for the selected xenoestrogens, were used (Appendix 1). In the main study, the exposures included nominal single concentrations of BPA, NP and OP at 500, 50 and 20 µg l^{-1} respectively, and of two mixtures, i.e. mixture 1 (Mix 1) with BPA 500 + NP 50 + OP 20 µg l^{-1} and mixture 2 (Mix 2) with BPA 250 + NP 25 + OP 10 µg l^{-1}. For both studies the solvent control (MeOH) and positive control (EE2, nominal 25 ng l^{-1}) were used for quantification of relative mRNA abundance and response. Fish were sampled at the end of five days of exposure. Each replicate (25 individuals; total biomass load being ca. 20 mg l^{-1} per day) was pooled as one analytical sample, collectively weighed in RNase-free micro centrifuge tubes (Starlab GmbH, Hamburg), and snap-frozen in liquid nitrogen and stored at –80 °C until further analysis.

3.4.3 Sediment-elutriate exposure with *V. fischeri* (III, IV)

Bioluminescence inhibition of *Vibrio fischeri* by sediment elutriates was measured on a Sirius single tube luminometer (Berthold Detection Systems GmbH) using a BioTox luminometric toxicity kit (Aboatox Oy, Finland, ISO, 1998; 2006). Samples were diluted with 2 % NaCl to constitute 5.6, 11.3, 22.5, and 45 % of the nominal 100 % sediment elutriates (1 + 4 v/v elutriates of HL, TBT-spiked, and SLS samples). The elutriates were prepared according to Hyötyläinen and Oikari (1999) and stored at 4 °C to be used in exposure assays within a week. The controls for the assay included diluent (2 % NaCl) and dissolved TBT-Cl. The samples were inoculated with equal volume of bacteria and initial light intensities were measured, and further incubated at 15 °C before measurements after 15 and 30 min. The inhibition (INH %) effect was calculated based on the relative bioluminescence before and after incubation according to the manufacturer's instructions. The toxicities and threshold concentrations (EC_{50}; effective concentration that reduces bioluminescence by 50 %) of the samples were determined.

3.4.4 Exposure of zebrafish pre-hatched embryonic life-stage (0–3 dpf) to OTC-contaminated sediment elutriates (III)

Fertilized eggs were collected from breeding tanks and transferred to the E3 embryo rearing medium in Petri dishes. Normally-developing embryos (1–2 hpf) were selected under an inverted microscope for the exposure and assay (III). Zebrafish embryos were exposed to 50 and 100 % dilutions of sediment elutriates, 1 + 4 (v/v), of PJ, HL and TBT-spiked samples for 72 h. Each exposure unit included 10 embryos in 10 ml assay solution (pre-aerated to 100 % saturation) in 25 ml beakers, repeated in triplicates for one exposure concentration (Fig. 9a). In addition to a water control, the PJ elutriate was considered as a reference since it was used as a TOC-source in the dilution and spiking of native sediments. The exposure containers were maintained in a Peltier-cooled incubator (Memmert GmbH, Germany).

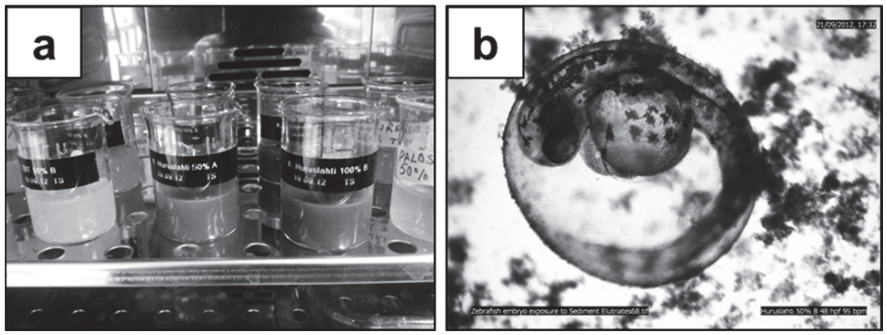


FIGURE 9 Zebrafish embryos exposed to water elutriate (a) of sediment from Lake Huruslahti (HL) contaminated with OTCs. (b) Zebrafish embryo (48 hpf) exposed to HL-50 elutriate. Note that fish embryos were in direct contact with sediment throughout the exposure.

The developing embryos inside chorion were observed under a stereo microscope (Leitz DM RBE, Leica, Germany) for morphological and pathological endpoints, including developmental abnormalities, heartbeat frequency, hatching rate and mortality (Fig. 9b). Heart rate was observed from six embryos (two per triplicate) per exposure, each observed for at least one min, keeping the conditions (light, temperature) stable throughout the observation procedure. After each observation, the embryos were transferred to a fresh exposure medium (100 % saturated DO). At the end of experimentation, the oxygen concentration (revealing > 70 % saturation) of the exposure medium was confirmed.

3.4.5 Whole sediment (OTC-contaminated) exposure with *20dpfZF* (III)

For one experimental unit, wet sediment and overlying water (3 °dH or 54 mg $CaCO_3$ l^{-1}, pH 7–7.2) were prepared in 600 ml glass beakers in a ratio 1:4 (dw/v). To avoid any suspension of particulates to the water layer, first ca. 80 g

of wet sediment was added whereafter a circular slab of frozen overlying water (100 ml; ca. 1 cm thickness), made to the diameter of the exposure beaker, was carefully placed on the sediment (using forceps) (Fig. 10a). Finally the remainder of the overlying water was slowly poured over the ice slab to get it afloat without disturbing the underlying sediment (Fig. 10b). The experimental units were covered and allowed to stand in the dark for ~16 h at 24–26 °C before the experimental fish (n = 15) were added (Fig. 10c).

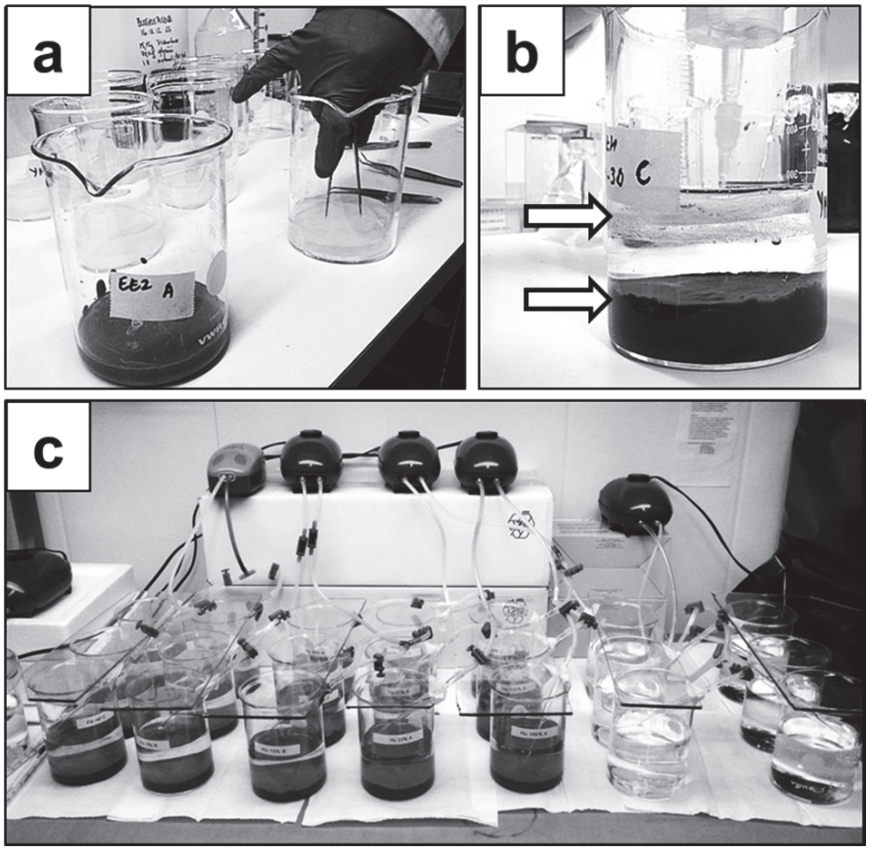


FIGURE 10 Preparation of an experimental unit for whole sediment bioassay with zebrafish, (a) circular ice slabs placed on ca. 80 g wet sediment, and (b) water poured over the ice slab to get it afloat over the underlying sediment as shown by arrows. (c) Aerated experimental units in series.

3.4.6 Whole sediment (PPM-contaminated) exposure with *20dpfZF* (IV)

The preparation of experimental units was similar to what has been described above. The 600 ml glass beaker exposure series consisted of control (RN, 0–10 cm), 1 km (0–5 cm, 20–30 cm sediment depth) and 3 km (0–5 cm, 20–30 cm), as well as of the EE2-spiked sediment (3 µg g^{-1}dw) as the positive control. The exposures of animals were conducted in gently aerated static systems (Fig. 10c)

without water renewal over 72 h, and sampled (n = 3 pools of 15 fish each) at the end of ~72 h from start of exposure. To maintain good water quality, the fish were not fed during the experiment. Dissolved oxygen (ppm) at the sediment-water interface was measured daily using a NeoFox Phase Measurement system (Ocean Optics, U.S.A.). At the end of exposure the pH of the overlying water and the sediment slurry was measured using a pH meter (PHM220, Radiometer Analytical).

3.5 Analytical methods

3.5.1 Determination of water concentrations of EE2, BPA, NP, and OP (I, II)

Ethinylestradiol was extracted from water samples by a Solid-phase extraction (SPE) method (Waters 2008) modified for the analyses (I, II). Similar extraction was employed with NP and OP samples (3–10 ml) using BPA-d16 as the internal standard. The gas chromatography–mass spectrometry (GC–MS) of EE2 concentration was performed with HP 6890 GC (Hewlett-Packard, Walbronn, Germany) equipped with HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, USA). The method included a derivatization by silylation with BSTFA and with pyridine to increase the stability of derivatized products and to improve extraction recoveries of the target compounds (Zhang *et al.* 2006). The actual experimental concentrations of chemicals were monitored during the study, and to do that the water samples (two replicates) were collected on days 1 (24 h) and 4 (96 h). Solid-phase extracts of EE2 (500–1000 ml), NP and OP (3–10 ml) were isolated from the samples. BPA-d16 and EE2-d4 were used as the internal standards.

3.5.2 Total organic carbon and OTCs in sediments (I, III, IV)

The total organic carbon (TOC % dw) determinations of sediment samples were performed before the whole sediment bioassays (I, III, IV). Dry weight was determined after oven-drying at 105 °C for 18 h. For loss on ignition (LOI), the dry samples were then burned at 550 °C for 2 h, the weight ratio (550 °C:105 °C) representing the inorganic solid residue (%) as LOI, an estimate of total organic matter. For TOC, samples were prepared according to Nikkilä *et al.* (2001) and ca. 2.6 mg of freeze-dried sediment was analyzed in a Thermo Finnigan EA 1112 Series Flash Elemental Analyser.

Organotin analysis of the HL and TBT-spiked sediment was performed in March, 2013 (III) and the information compared with the analysis performed in February 2012 in order to determine any difference in the TBT of further aged HL sediment. Analysis was repeated to confirm similarity of contamination of TBT in HL sediment used in assays conducted at different time points. Wet sediment samples were sent to the Institute for Environmental Research (YMTK, an accredited laboratory) of the University of Jyväskylä for chemical

analysis, revealing data of February 2012 (HL-100*) and March 2013 (PJ, HL-10, HL-100, TBT-10, TBT-100) (III).

3.5.3 Real-time qPCR

Total RNA was extracted from homogenized pooled fish samples (15 animals with total weight ca. 16 mg) using the Tri Reagent (Molecular Research Center, Inc.) according to the manufacturer's instructions, and the quality and quantity were determined on a Nano Drop ND-1000 UV-visible spectrophotometer. Absorbance measurements including 260:280 ratios (1.8 to 2.0) and the additional 260:230 > 260:280 ratios indicated a secondary measure of nucleic acid purity (Nano Drop 2007). The integrity of RNA was also verified using the Agilent RNA 6000 Nano Assay Protocol with an Agilent 2100 Bioanalyzer (Waldbronn, Germany). RNA integrity number (RIN, 1–10) generated for analyzed samples was checked to comply within the range of 7–10 for all samples. The total RNA extracted was DNase-treated and reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, USA), and the cDNA templates were diluted ten times for use in real time PCR assays.

Real-time PCR was performed using iQ SYBR Green supermix (Bio-Rad, USA) and run on a Bio-Rad CFX96™ real-time PCR detection system. Gene-specific primers (Table 1) for house-keeping genes (beta-actin *β-actin* and elongation factor 1 alpha *ef1a*) (I–IV) as well as target genes (*cyp1a1*, cytochrome P450 1A1 (III, IV); *cyp11a1*, P450 side chain cleavage (I); *cyp19a1b*, brain aromatase (I–IV); *ef1a*, elongation factor 1 a (I–IV); *esr1*, estrogen receptor 1 (II); *hsd3b1*, 3-beta-hydroxysteroid dehydrogenase (I); *star*, steroidogenic acute regulatory protein (I); *vtg1*, vitellogenin 1 (I–IV) were designed using Amplifx (v 1.5.4). The primer pair sequences were checked with the program Mfold to predict the potential for amplicon secondary structures that may prevent efficient amplification (Zuker 2003). To evaluate qPCR efficiency, amplification efficiency with standard curve analysis (PCR efficiency, 90–110 %; slope, –3.1 to –3.6; $R^2 > 0.98$) was performed, and optimization of qPCR reactions and protocol was carried out.

The qPCR reaction mix was optimized for a 25 µl reaction mix consisting of 12.5 µl of SYBR green supermix, 2.5 µl of each of the primer pairs (200 nM), 2.5 µl sterile water, and 5 µl of cDNA template (100 ng μl^{-1}). The PCR protocol comprised 10 min polymerase activation at 95 °C, followed by 40 cycles of 30 sec at 95 °C, and 30 sec at 60 °C. Each sample was assayed in duplicate, and assays requiring multiple runs included inter-run calibrators (IRC) on each of the plates to facilitate sample maximization and to restrict reference gene reactions to a single plate. The IRC was performed to correct for variation, allowing comparison between plates within the same analysis.

TABLE 1 Primer pair sequences, amplicon sizes, and accession numbers for genes investigated in this thesis. *β-actin*, beta-actin (I–IV); *cyp1a1*, cytochrome P450 1A1(III, IV); *cyp11a1*, P450 side chain cleavage (I); *cyp19a1b*, brain aromatase (I–IV); *ef1a*, *Danio rerio* elongation factor 1 a (I–IV); *esr1*, *Danio rerio* estrogen receptor 1 (II); *hsd3b1*, 3-beta-hydroxysteroid dehydrogenase (I); *star*, steroidogenic acute regulatory protein (I); *vtg1*, vitellogenin 1 (I–IV).

Gene	Primer sequences (5′-3′)	Size (bp)	GenBank accession
β-actin	Forward-AAGAGCTATGAGCTGCCTGA	108	AF057040.1
	Reverse-ACCGCAAGATTCCATACCCA		
cyp1a1	Forward-AAG TTG GAA GGC GAG AAG GTG	98	AF210727.2
	Reverse-ATG GCC AGG AAC AGG AAG ACT		
cyp11a1	Forward-GCCTGGAAGACTGATAGGATGA	114	AF527755.1
	Reverse-GTTGACACGAGCCACAAAGT		
cyp19a1b	Forward-TGGACGCATGCATAAGACAG	104	AF226619.1
	Reverse-CACAACCGAATGGCTGGAA		
ef1a	Forward-AAACATGGGCTGGTTCAAGG	87	AY422992.1
	Reverse-ATGGCATCAAGGGCATCAAG		
esr1	Forward-TCGATTTCGGAGTCTCAAGC	131	NM_152959.1
	Reverse-ACTGCACCATGAAGTTGTCC		
hsd3b1	Forward-AGCCCATTCTGCCCATCTTT	120	AY279108.1
	Reverse-CCATGGTCACTAGCTGCCTATT		
Star	Forward-TGTAAGGGCTGAGAATGG	133	NM_131663.1
	Reverse-TACTCGGTTGATGACAGTT		
*vtg1**	Forward-GCTTTGCCTATTCCCACATC	136	NM_001044897.2
	Reverse-GCTCTGCTGTAACGGTAGT		

* Primer design for *vtg1* was custom ordered from Sigma-Aldrich.

3.6 Statistics

The normality of datasets was analysed by the Shapiro-Wilk's W test and homogeneity of variance was checked by Levene's test (I–IV). Non-normally distributed data were $\log_{10}$-transformed prior to further analyses (I–IV). Based on Levene's test, data that met the assumption of homogeneity of variance were analysed with one-way analysis of variance (ANOVA). Heteroscedastic datasets that did not meet the homogeneity of variances assumption were analysed with Welch's ANOVA. Corresponding to the analysis by either one-way ANOVA or Welch ANOVA, multiple comparisons between test groups were analysed with Tukey's HSD or Games Howell *post-hoc* tests, respectively (I–IV).

The expressions of target genes were normalized to the expression of two reference genes, *β-actin* and *ef1a*. The choice of reference genes was based on

their stable expression across samples of different treatments. The determined stability parameter values (M-value) to select suitable reference genes with M-values were < 0.5; the lower the M-value, higher the stability (Vandesompele *et al.* 2002). The gene expression levels were calculated from the experimental amplification data (Cq values; quantification cycles) using the Pfaffl method (Pfaffl 2001) relative to the controls.

Steroidogenic enzyme (*cyp11a1*, *cyp19a1b*, *hsd3b1* and *star*) mRNA levels in juvenile zebrafish exposed to water-borne EE2 (I) as well as *cyp19a1b* and *vtg1* data from exposure to EE2-contaminated sediment via water (I) were analyzed by one-way ANOVA and Tukey's HSD *post-hoc* test. In the same experiment series, exposures to water-borne xenoestrogens (BPA, EE2, NP, OP) change in *cyp19a1b* and *vtg1* transcripts was determined using the Welch ANOVA and Games-Howell *post-hoc* test. In water-borne exposure to xenoestrogens as mixtures (II), *cyp1a*, *cyp19a1b* and *esr1* transcription data were analysed by one-way ANOVA and Tukey's HSD *post-hoc* test, whereas differences in *vtg1* were determined using the Welch test followed by the Games-Howell *post-hoc* test.

In the sediment studies where zebrafish embryos were exposed to elutriate of OTC-contaminated sediment (III), difference of heart rate between groups was determined using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. For relative gene quantification in *20dpfZF* used in whole sediment assay, SC (0.01 % MeOH solvent control) and PJ were used as controls corresponding to water-borne and sediment exposures, respectively. The gene induction data for *vtg1* and *cyp19a1b* of HL-sediment assay were analyzed using Welch's ANOVA followed by Games-Howell *post-hoc* test. Data (*cyp19a1b* and *vtg1*) from TBT-spiked sediment assay was determined by one-way ANOVA followed by Tukey's HSD *post-hoc* test. In the SLS sediment assay with *20dpfZF* (IV), data (*cyp19a1b*) from SLS sediment assay was determined by one-way ANOVA followed by Tukey's HSD *post-hoc* test, while *cyp1a* and *vtg1* were analyzed using Welch's ANOVA and multiple comparisons made using Games-Howell *post-hoc* test.

In all studies the significance level of 0.05 was set to indicate a difference between means of experimental groups. All analyses were performed using IBM SPSS 19.0 statistical package (IBM Corp, USA).

4 RESULTS

4.1 Water analyses

4.1.1 Water–borne concentration of xenoestrogens

No significant differences in water quality, like oxygen, pH, and temperature, were found between experimental jars or treatments during the exposure periods. Compared to nominal concentration added in experimental waters, actual chemical concentrations (mean ± SD) were lower (I). Most unexpectedly, all measured concentrations of NP were not detectable. Since both NP and OP in samples were extracted simultaneously with the same method, the undetected NP may have resulted from some undefinable issues with the extraction method. For clarity, while the measured concentrations commonly ranged 20–50 % below expected, revealing dose-related effects, the nominal values are used in further discussion.

In Study II, single exposures to BPA, NP and EE2, the measured values corresponded to around 76 % (NP), 89 % (EE2) and 105 % (BPA) of nominal concentrations. The measured concentration of BPA and NP in the mixture exposure (Mix 1) ranged, however, from 26 % (24 h) to 1 % (96 h), and 7 % (24 h) to 12 % (96 h) of nominal, respectively. On the other hand, in the case of measured concentrations for Mix 2, BPA showed higher proportions, from 56 % (24 h) to 138 % (96 h) of nominal levels. As in Mix 1, measured NP ranged from 12 % (24 h) to 24 % (96 h) of nominal. The measured concentrations of OP showed the lowest values (6 %) of nominal for the highest concentration of individual chemical exposures and even below detection for others. Values from the first series of OP exposure (I) measured concentrations ranged from 7 to 31 % of nominal. Despite non-constant exposure levels to many xenoestrogens, daily renewable of the media, by administering chemical stock up to nominal working concentrations based on a routine protocol, ensured relative differences between exposure settings.

4.1.2 Sediment characteristics and quality

The reference sediment of Lake Palosjärvi, PJ, as a native sample showed high total organic carbon (TOC = 19.5 % dw) compared to the reference RN at the SLS study area (Table 2) (I, IV). For sediment sampled from SLS in October 2011 (IV), the TOC (%) of the RN reference sediment was lower (5.5 % dw) compared to the downstream sites (1 km, 0–5 cm: 12.2 %, 5–10 cm: 14.4 %; 3 km: 0–5 cm: 11.8 %, 5–10 cm: 14.6 %). In Study III, the mean dissolved oxygen concentration in water in experimental units ranged from 7.8 to 9.3 mg l^{-1}. At the end of 3-day exposures, the pH values of sediments (pH 5.2–5.5) appeared somewhat more acidic than in the overlying waters (pH 5.3–6.3) (Table 2).

TABLE 2 Properties of sediments used for animal exposures in studies I, III and IV. PJ, reconstituted reference sediment from Lake Palosjärvi; HL, Lake Huruslahti; RN, Lake Rautniemi.

Sediment source	Sediment pH	Overlying water pH	Dry weight (%)	Inorg. solids (%)	LOI (%)	TOC (%)	TBT (µg kg^{-1}dw)
PJ	5.2	6.3	19.1	89.0	11.0	4.3*	nd
HL	5.2	5.4	16.0	88.3	11.7	4.3	67.0
RN	5.0	-	12.8	85.8	14.2	5.4	-

LOI = loss on ignition; TOC = total organic carbon; nd = not detected (< LOD, limit of detection).
* nominal total organic content (ca. 4.3 %) based on addition of inorganic constituents to the native PJ sediment (TOC= 19.5 %) to form reconstituted PJ sediment.

The chemical analysis of the pooled HL sediment investigated (Table 3) (III) showed TBT as the largest proportion of the overall OTC load. The maximal concentrations of MBT, DBT and TBT in the vicinity of the sampling site chosen in this study, as reported before (Anon 2009), can be 75–140 times that found in our pooled HL sediment. The butyltin degradation index, (MBT + DBT)/TBT = 0.4, being less than 1, suggested a relatively recent TBT contamination (Diez *et al*. 2002). Importantly, although the HL 100 % (undiluted) sample represented a lower concentration compared with earlier maxima, the HL sediment investigated represented anticipated and possible impacts much beyond the most OTC-contaminated sub-area in Lake Huruslahti and downstream (Anon 2009). While the TBT concentration in the HL sample was less than we anticipated, the surrogate concentration in TBT-spiked sediment (TBT-100, Table 3) (III) was set to the upper range found earlier in HL, this being 24 times that in the HL pool collected in 2011.

TABLE 3 Organotin and organochlorine analysis of the pooled surface sediment (0–10 cm depth) used in the study (III). The samples designated are PJ (Lake Palosjärvi), HL (Lake Huruslahti) and TBT-spiked sediment, the numbers indicating percentage dilution of the most strongly contaminated sample (100 %) of HL and TBT-spiked sediments, respectively.

Chemical compounds (µg kg-1) dry. wt.	Sediment samples					
	HL-100 [a]	PJ [b]	HL-10 [b]	HL-100 [b]	TBT-10 [b]	TBT-100 [b]
Monobutyltin (MBT)	4.8	nd	0.6	3.0	6.5	79.0
Dibutyltin (DBT)	25.0	nd	1.7	21.0	35.2	344.0
Tributyltin (TBT)	61.0	nd	5.0	67.0	185.0	1620.0
Tetrabutyltin (TTBT)	nd	nd	nd	1.9	2.8	16.0
Monophenyltin (MPhT)	4.9	nd	< 1.0	3.0	0.6	7.3
Diphenyltin (DPhT)	nd	nd	nd	nd	nd	nd
Triphenyltin (TPhT)	nd	nd	nd	nd	nd	nd
Mono-n-octyltin	nd	nd	nd	nd	nd	nd
Di-n-octyltin	nd	nd	nd	nd	nd	nd
Tri-n-octyltin	nd	nd	nd	nd	nd	nd
PCBs (mg kg^{-1}) [c]	< 0.002	na	na	na	na	na
PAHs (mg kg^{-1})	< 0.1	na	na	na	na	na
PCDD/F (µg kg^{-1})	< 0.03	na	na	na	na	na

nd = not detected; na= not analysed.
a = LOD was 1 µg kg^{-1} for 2012 data, measured from the pooled HL sample;
b = LOD was 0.5 µg kg^{-1} for 2013 data, measured after animal exposures for 72 h;
c = Data for PCB-sum of congeners 8, 18, 28, 52, 101, 105, 118, 128, 138, 153, 156 and 180.

The composition of effluents and contaminated sediments in the Lappeenranta area of SLS highlights the presence of resin acids (RAs), betulinol, and wood sterols (WSs), possibly acting on fish reproduction and endocrinology (IV). Spatially and temporally the concentration of RAs and WSs show some variability, revealing an overall gradient in levels of contamination over the sites (i.e. 1 km > 3 km, Lahdelma and Oikari 2006; Table 4). Further, the proportion of dehydroabietic acid (DHAA) has been consistently the most abundant RA found deposited in receiving sediment. The concentration of β-sitosterol, a major sterol in PPME discharges, corresponded with the concentration gradient of total WSs at 1 and 3 km downstream from the mill, which was to be 35 and 5 times that in the upstream reference site (Lahdelma and Oikari 2006; Table 4). The relative differences between gene-probed sediments are thus obvious (IV), and maintained over five decades or more, although not reanalysed for the given study.

TABLE 4 Observations on chemicals in sediments (0–10 cm) from downstream (1 km, 3 km) and upstream (Lake Rautniemi, RN) sites from the chemical wood processing mill in Southern Lake Saimaa. Sample cores were collected in 2001 (Lahdelma and Oikari, 2005, 2006) and 2010 (Ratia *et al*. 2014) and represent the same sampling site as studies (IV). For relative comparison, concentrations from 0–5 and 5–10 cm were averaged to obtain an approximate combined concentration of 0–10 cm sediment core sampled in 2001.

Compound	RN (reference)		1 km		3 km	
(μg g^{-1} dw)	2001	2010	2001	2010	2001	2010
β-sitosterol	25	-	860	-	115	-
Retene	0	0	125	224	27	94
Dehydroabietic acid	-	1.1	-	57	-	54
Total resin acids	10	1.4	675	135	430	170
Total wood sterols	44	-	1655	-	275	-
TOC %(dw)	5.3–6.5	-	17.5–19.0	-	10.8–18.2	-

- = no data available; 0 = < LOD

4.2 Selection of responsive molecular biomarkers in *20dpfZF* to probe xenohormonal contamination

4.2.1 Selection of reference genes for expression analyses

To ensure reliable quantification of target genes, two reference genes, *β-actin* and *ef1a,* were assessed for their stable expression across a range of chemicals and their concentration (I). The mean M–values of both reference genes across the corresponding concentration series of exposures (BPA, NP, OP, and EE2) were 0.004, 0.1, 0.3, 0.06; and 0.01, 0.04, 0.06, 0.09 respectively, showing stable expression of the respective reference genes (Fig. 11). For normalization of gene expression, the mean M–values of both reference genes combined was considered together for the chemical exposures, and were 0.01, 0.07, 0.06, and 0.07 respectively. Values suggest stable expression of this reference gene pair across chemical treatment at the studied life-stages (I).

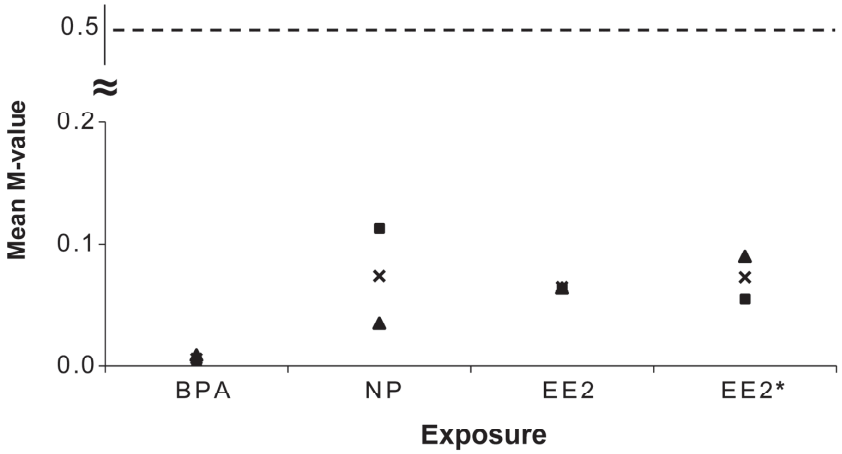


FIGURE 11 Stability of reference genes in whole-body homogenates of *20dpfZF* exposed to common xenoestrogens (I). Lines with markers (■) represent mean M–value for *β-actin* across concentrations in chemical exposures. Lines with markers (▲) represent mean M–value for *ef1a* across concentrations. Lines with markers (×) represent mean M–value for housekeeping genes *β-actin* and *ef1a* across concentrations in chemical exposures. The dotted line indicates the recommended stability threshold M–value = 0.5 (Vandesompele *et al.* 2002).

4.2.2 Selection of genes impacted by steroidogenic challenges

To select key genomic signals in the steroidogenic pathway accompanying endocrine modulation, *20dpfZF* were exposed to EE2, a potent synthetic estrogen, for five days (20–25 dpf) (I). Among the normalized gene expression levels of *cyp11a1*, *cyp19a1b*, *hsd3b1*, and *star,* only *cyp19a1b* showed pronounced upregulated expression levels (Tukey's HSD, $p < 0.001$). Noticeable induction of *cyp19a1b* resulted from three EE2 exposures (5, 25 and 50 ng l^{-1}), averaging 14, 19 and 23-fold changes respectively, with gradual increase with increasing concentration (5–50 ng l^{-1}). There was no induction of *star* in exposure groups, with expression even in the highest EE2 concentration, 50 ng EE2 l^{-1}, being similar to control and 5 ng EE2 l^{-1}. Although 25 ng EE2 l^{-1} induced ~1.3-fold expression of *star*, it was not significantly different from either the control or respective expression levels of *cyp11a1* and *hsd3b1* (Tukey's HSD, $p > 0.05$). More specifically, there was a statistically significant difference between responses of *cyp19a1b* and the other steroidogenic enzymes (*star*, *cyp11a1* and *hsd3b1*) as determined by one-way ANOVA for EE2 exposures 5 ng l^{-1} (F (3, 8) = 91.740, $p < 0.001$), 25 ng l^{-1} (F (3, 8) = 61.130, $p < 0.001$) and 50 ng l^{-1} (F (3, 8) = 133.082, $p < 0.001$). A Tukey's HSD *post-hoc* test revealed the significant up-regulation of only *cyp19a1b* expression among the studied steroidogenic enzymes ($p < 0.001$) compared to *star*–*cyp11a1* ($p = 0.848$) and *star*–*hsd3b1* ($p = 0.982$), thus making aromatase the key biomarker to assess xenoestrogenicity in zebrafish ELS (I–IV).

4.3 Modulation of estrogen-responsive gene transcripts in *20dpfZF*

4.3.1 Impact of water-borne exposure to xenoestrogens on *cyp19a1b* and *vtg1* transcript levels

The aim was to evaluate the estrogen-responsiveness of *cyp19a1b* and *vtg1* in *20dpfZF* when exposed to model chemicals (BPA, EE2, NP and OP) with a range of xenoestrogenic potency (I). The mRNA levels of *cyp19a1b* exposed to 5, 25, and 50 ng EE2 l^{-1} were induced to 13, 19, and 24-fold respectively, relative to control treatment (Fig. 12a). For the same concentrations, relative expression levels of *vtg1* were 5, 143, and 1272-fold. Despite the larger extent of *vtg1* expression across the exposure treatment, *cyp19a1b* showed higher sensitivity (13-fold; Games-Howell, $p < 0.05$) at the lowest range (5 ng EE2 l^{-1}) compared to *vtg1* (5-fold; $p < 0.05$). As presumed, the expression pattern of *cyp19a1b* at all concentrations of EE2 was similar to samples studied for steroidogenic enzyme mRNA levels (I); with a gradual increase from 5 to 50 ng EE2 l^{-1}. However, at higher treatments (25 and 50 ng EE2 l^{-1}), the relative induction of *vtg1* (Games-Howell, $p < 0.01$, 0.001) was noticeably higher than *cyp19a1b* (Games-Howell, $p < 0.05$), albeit that both genes showed significant relative expression. Almost 15 % mortality was observed in groups exposed to the highest nominal concentration of EE2, perhaps implying the lower range of sublethality.

Whole-body *cyp19a1b* showed increased expression levels at 100, 500 and 1000 µg BPA l^{-1} compared to *vtg1* (Fig. 12b). Expression of *vtg1* was down-regulated (in average –1.2-fold change) by lower concentrations of BPA (100 and 500 µg l^{-1}), but was significantly up-regulated at the highest concentration (2.7-fold change; Games-Howell, $p < 0.05$). The two highest concentrations of BPA l^{-1} showed significant induction of *cyp19a1b* transcripts, 2.9 and 4.4-fold (Games-Howell, $p < 0.05$) respectively. Clearly, again, *cyp19a1b* was more sensitive compared to *vtg1* across a range of BPA concentrations, further revealing that BPA induces only a weak estrogenic effect when compared to EE2. In *20dpfZF* exposed to 10, 50 and 100 µg NP l^{-1}, the relative expression levels of both *cyp19a1b* and *vtg1* showed no statistically significant change (Games-Howell, $p > 0.05$), demonstrating a tendency for down-regulation of both genes (Fig. 12b).

Compared to NP, the expression pattern with OP exposure was different; *cyp19a1b* showed an up-regulatory pattern for all three concentrations compared to control (Fig. 12b), the relative induction of being statistically significant only at 100 µg OP l^{-1} (2.5-fold; Games-Howell, $p <0.05$). For all the concentrations, again *cyp19a1b* showed higher sensitivity than *vtg1* for the induction, both 50 and 100 µg OP l^{-1} affecting the survival of exposed fish to ~20 and 30 % mortality (data not shown), respectively, implying the approach of the sub-acutely lethal range. Overall it is noteworthy that NP compared to EE2, BPA, and OP had a much less estrogenic effect in the *20dpfZF* model. In all,

from the exposure series with *20dpfZF*, a ranking of the estrogenic potency was obtained, with EE2 being the most potent agonist, followed by OP, BPA, and NP, in that order.

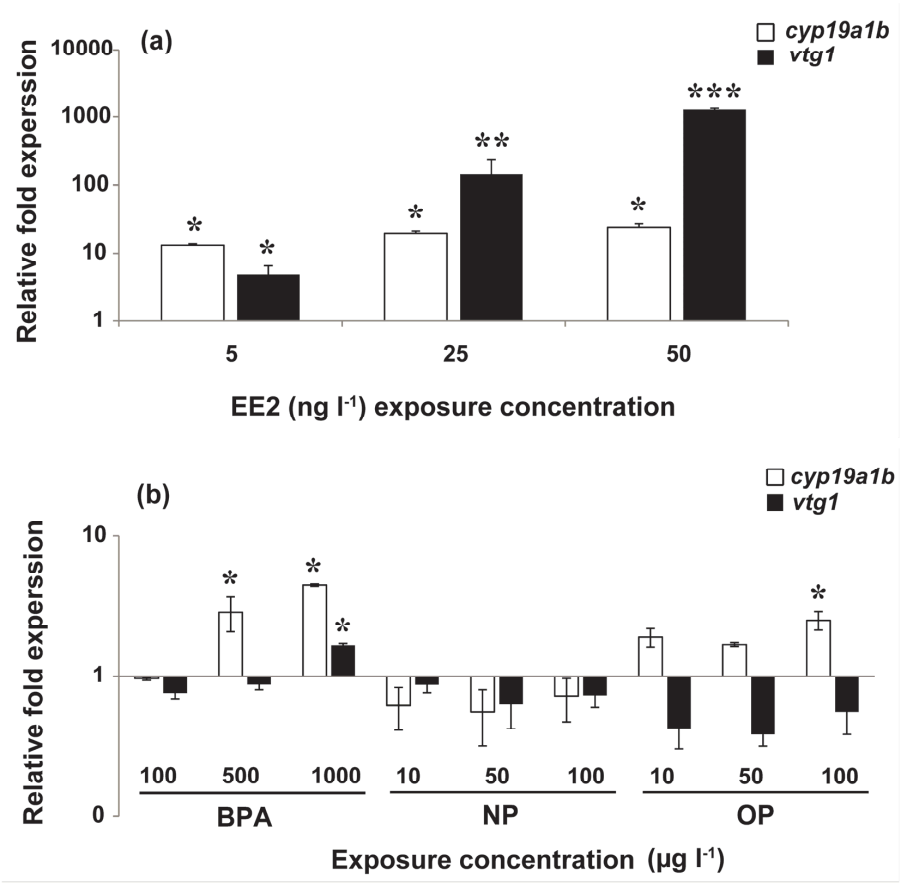


FIGURE 12 Xenoestrogen-induced gene expressions in early-juvenile zebrafish *20dpfZF* (I). Fold-change expression of *cyp19a1b* and *vtg1* in *20dpfZF* exposed to three nominal concentrations of EE2 (a), BPA, NP and OP (b) for five days i.e., over the period 20–25 dpf, in a whole-body assay (I). Bars represent gene expression fold-change (± SD; n = 3 pools of 15 fish each) normalized to geometric mean of *β-actin* and *ef1a* and relative to solvent (0.01 % methanol) control. The expression levels are in $\log_{10}$ scale. Note that expression of both *cyp19a1b* and *vtg1* genes were down-regulated in NP exposure (b). The asterisks represent statistically significant differences, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as determined by Welch's test followed by Games-Howell *post-hoc* test.

4.3.2 Mixture effects of XEs on *cyp19a1b*, *esr1* and *vtg1* transcript levels

In Study II, the estrogen-responsiveness of model chemicals (BPA, NP and OP) was assessed using gene expression analyses of *cyp1a*, *cyp19a1b*, *esr1* and *vtg1*

when present as single chemical as well as of two fixed ratio combinations (Fig. 13). Along the given setting, the gene induction pattern of *20dpfZF* after exposure to fixed combinations of BPA + NP + OP was compared to the approximate NOEL-effects of individual chemicals to assess the combinational impacts (Fig. 3).

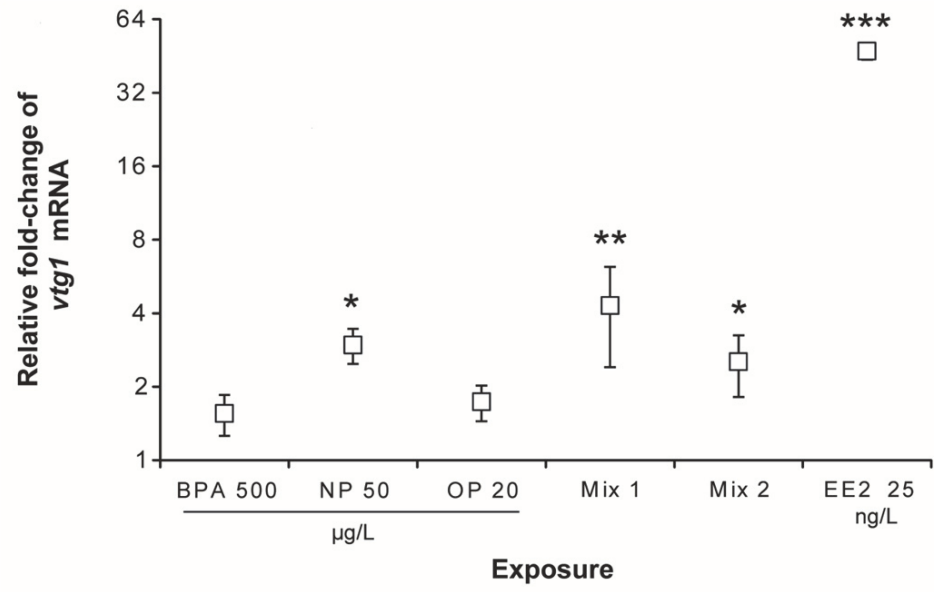


FIGURE 13 Relative gene transcription of *vtg1* in early-juvenile zebrafish (*20dpfZF*) exposed to series of exposures of BPA, NP and OP and their fixed ratio mixtures (Mix 1, Mix 2) over a five-day exposure period (II). Individual chemical concentrations for the mixture exposures were selected based on the non-significant gene induction of *vtg1* (I). Boxes represent mean ± SD for pooled whole-body homogenates (n=3 each with 25 pooled individuals). EE2 is the positive control treatment. Statistical significance ($p < 0.05$) was determined with Welch's ANOVA for *vtg1* (F (5, 5.105) = 696.982, $p < 0.001$) followed by Games-Howell *post-hoc* test. The asterisks represent statistically significant differences, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The gene expression responses of the mixture exposures (Fig. 13) revealed that expression of *vtg1* in this series was up-regulated by most test exposures; strongest with NP ($p < 0.05$). The dissimilar response to NP in Study I (Fig. 12) and Study II (Fig. 13) could be due to variation between different batches of *20dpfZF*, which were temporally and nutritionally separate. Also the dose of NP by water may have varied. The positive control, EE2 expectedly showed highly significant ($p < 0.01$) induction of both *cyp19a1b* (7-fold) and *vtg1* (47-fold) genes. Thus, this time, *vtg1* was comparatively more sensitive than *cyp19a1b*, with statistically significant fold changes observed at both mixture treatments (Mix 1, 2) (II). While BPA (500 µg l^{-1}) and OP (20 µg l^{-1}) resulted in statistically non-significant fold change, NP (50 µg l^{-1}) showed significant up-regulation also of *cyp19a1b*, not only that of *vtg1* gene. Furthermore, a statistically significant

difference in induction of *vtg1* mRNA between NP and Mix 1 (3.2-fold vs 4.3-fold, Fig. 13) indicated a concerted effect, and one induced not by the impact of NP alone.

Importantly as well, *vtg1* was significantly up-regulated (2.5-fold) with the exposure to Mix 2, which was the half nominal concentrations of Mix 1, revealing above-additive effect (Fig. 13). Overall, *vtg1* was up-regulated in early juvenile fish exposed to mixtures relative to individual concentrations of the selected xenoestrogens, suggesting in minimum the additive effect of low-dose xenoestrogens *in vivo*.

4.4 Toxicity of xenohormonal compounds in the sediment – Response of embryonic and early juvenile life-stages of zebrafish

4.4.1 Toxicity of OTC-contaminated sediment elutriate on bioluminescence by *V. fischeri*

To evaluate the gross toxic potency of the OTC-contaminated sediment, *Vibrio fischeri* were exposed to elutriate prepared from naturally- and artificially-contaminated sediments (III). Impairment of bacterial energetics indicated by bioluminescence assay was evident from both sediment elutriates, which contained TBT and pure TBT. The mean EC50values (± SD) for TBT-Cl after 15 and 30 min assays were 17.7 ± 2.3 and 20.1 ± 2.9 μg l^{-1} respectively. Among the sediment elutriates (1 + 4 v/v) by this assay, HL-100 showed at least three inhibition values between 10–90 %, so the calculation of EC50 was possible using the gamma model. The EC50 values (% of HL-100 elutriate) were 52 ± 9.6 (15 min) and 35 ± 1.6 (30 min), respectively, revealing a toxic potency to interfere with the cellular energy chain function. Although it was not possible to calculate EC50 for elutriates made from TBT-100, the inhibition obtained for 45 % elutriate, the highest assayed, also represented a distinct EC-potency for that source of chemical contamination. The EC-value was lowest for PJ reference (III).

4.4.2 Embryotoxicity of OTC-contaminated sediment elutriate

The aim was to assess the toxic potency by elutriate of OTC-contaminated (natural and spiked) sediment on developing unhatched zebrafish embryos from post-fertilization until the hatched stage. Altered blood circulation (heart rate) (Fig. 14) and developmental deformities, including delayed development, edemas of the pericardial and yolk sac regions (Fig. 15) were frequently seen in exposures both to HL and to TBT-spiked sediment elutriates (III). One-way ANOVA showed statistically significant differences in heart rate between zebrafish embryos exposed to control and OTC-polluted elutriates at 24 h (F(6,

14) = 5.746, p = 0.003), 48 h (F(6, 14) = 2.978, p = 0.043) and 72 h (F(6, 14) = 2.911, p = 0.047) (Fig. 14). Duncan *post-hoc* comparisons indicated that, after 24 h, only embryos exposed to TBT-100 elutriate showed a significant decrease in heart rate compared to the control (p < 0.05), though TBT-100 also differed from responses caused by PJ-50, HL-50 and PJ-100 elutriates. After 48 h and 72 h, however, the only elutriates that caused a decrease in heart rate compared to the controls were TBT-50 and HL-100, respectively, further indicating the sublethal embryotoxicity of TBT-contaminated sediment from the industrially polluted Lake Huruslahti.

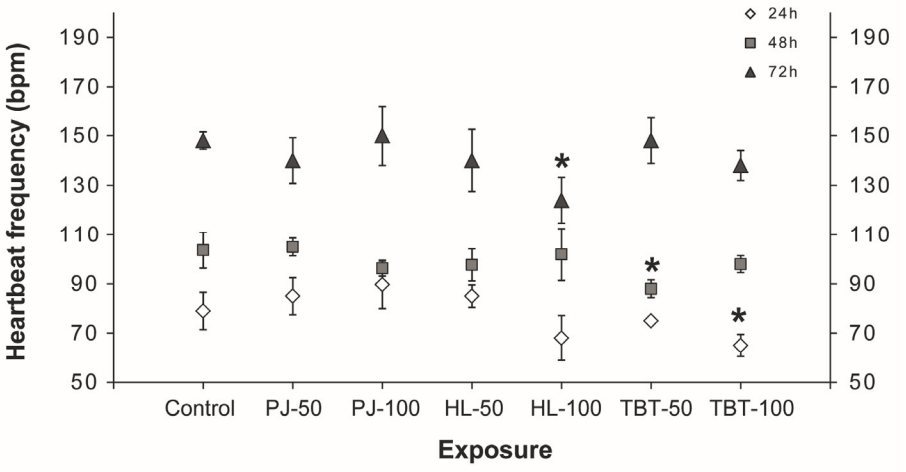


FIGURE 14 Effect of exposure to water elutriate on heart frequency (bpm, beats min^{-1}) in in 1–3 dpf embryonic zebrafish (III). Zebrafish embryos were exposed for 24, 48 and 72 h to control (water) and sediment elutriates of PJ (Lake Palosjärvi), PJ-50, PJ-100; HL (Lake Huruslahti), HL-50, HL-100; TBT-spiked sediment, TBT-50, TBT-100. Graph represents means (± SD) of heart rate observed for six embryos (n = 6). Asterisks represent significant differences (p < 0.05) between groups when compared to control.

The survival rate of embryonic and unhatched zebrafish was not affected by exposure to any elutriate concentrations of HL and TBT-spiked sediments (> 90 % survival) nor to the reference PJ (III). The few random mortalities (8–9 % at 72 h) observed were in exposures HL-50 and TBT-50. Instead some ontogenic abnormalities evolved in HL and TBT-spiked sediment elutriates relative to the water control across developmental time (Fig. 15). Developmental deformities included delayed development, edemas of the pericardial and yolk sac regions, and altered blood circulation (heart rate) were frequently seen in exposures of both HL and TBT-spiked sediment elutriates, assessed semi-quantitatively (III). The statistics of differences from six animals per exposure group were not determined, because of obstructed visualization by the sediment debris, not present in controls, adhering to some chorions that could not be rinsed away without disturbing the embryos.

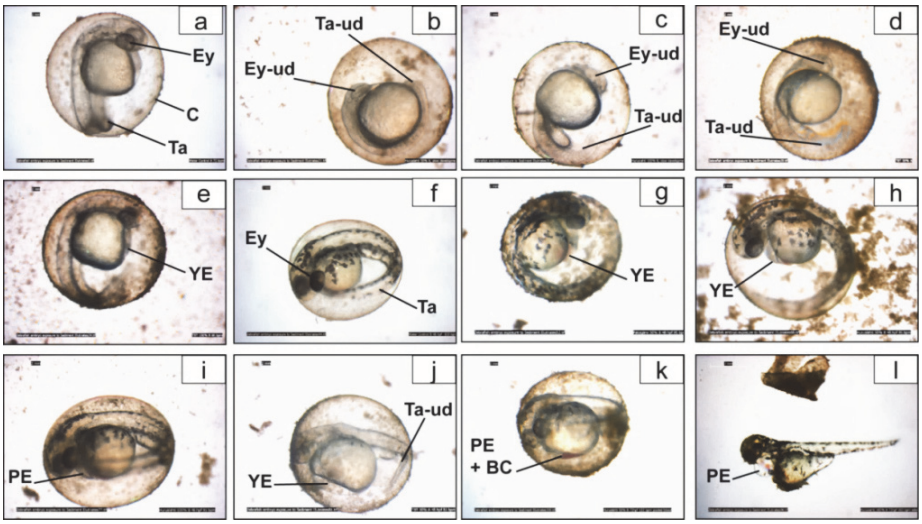


FIGURE 15 Developmental abnormalities in zebrafish embryos (1–3 dpf) exposed to sediment elutriate (III). Observation time-points were 24 h (a–e), 48 h (f–j) and 72 h (k, l) post-fertilization of zebrafish. (a) shows normal embryo (water control) after 24 h; (b), (c) and (d) revealing underdeveloped embryos in HL- (50 and 100 %) and TBT-50 exposures, respectively; (e) shows an early yolk sac edema (TBT-100 elutriate); (f) normal control embryo after 48 h; (g) and (h) show yolk sac edema in PJ-50 and HL-50 exposures, respectively; (i) showed pericardial edema in HL-100 exposures; (j) yolk sac edema and underdeveloped tail in TBT-50 exposure; and (k) and (l) showed pericardial edema in two HL-exposures (50 and 100 %), with blood pooling or coagulation shown in (k). (l) shows hatched zebrafish fry at 72 h. BC, blood coagulation; C, chorion; Ey, Eye; Ey-ud, eye underdeveloped; PE, pericardial edema; Ta, Tail; Ta-ud, tail underdeveloped; YE, yolk sac edema. Dimensions of embryos (diameter, 1.3–1.6 mm) and hatched fry (body length, 4–4.5 mm) were recorded. Note the debris attached to or in vicinity of embryos representing particulates formed from the exposure sediment elutriates.

4.4.3 Relevance of *20dpfZF* in whole sediment bioassays

The objective was to evaluate a life-stage incorporating practical and experimental advantages for screening environmental estrogens in contaminated sediment (Fig. 16) (I, III, IV). The zebrafish of the life-stage investigated (20 dpf) were found to be free-swimming and actively foraging in surface sediment (Fig. 16b) (I). While each group of 25 animals in the water-only units appeared to distribute randomly across the water column, the fish in experimental units with sediment headed for the sediment-water interface, perhaps seeking food (Fig. 16b, c).

This observation is not surprising, as by 5–7 dpf the yolk is depleted, and foraging behaviour of the fish determines their growth with trophic transfer of contents and, consequently survival. In the present study, zebrafish (6–8 mm) were reared from post-hatch (3 dpf) until 20 dpf were maintained in aerated glass trays (ca. 30 mm water depth). Fish were also reared in taller beakers,

allowing more water depth. Upon the addition of dry powdered food to either vessel, the food particles spread on the water surface in a manner dependent on the dimension of the particular container. In a glass tray, with shallow depth, almost all fish from rearing batches with density as high as 150 animals per litre get ready access to food. The increased water depth in beakers, however, favoured the first and most frequent feeders that were noticed to be the fittest and healthiest of the lot. For such reasons, it appears that post-hatch (3 dpf) fish that do not feed in the first few feedings, are not active enough to effectively cover relatively longer vertical distances, over subsequent feeding regimes. Therefore, *20dpfZF* model was technically feasible for food-borne exposures under controllable laboratory conditions.

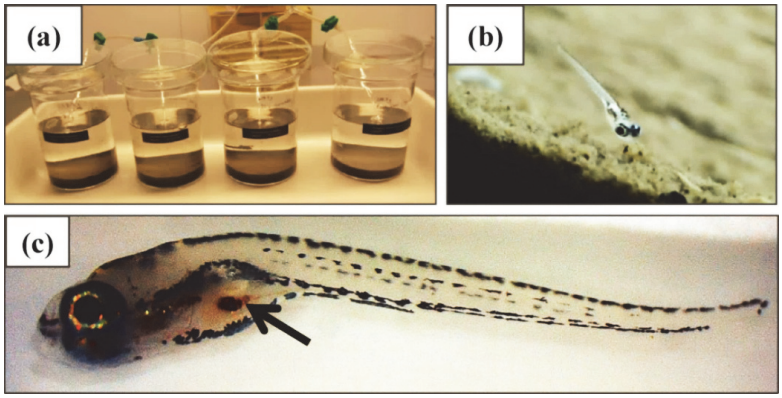


FIGURE 16 Experimental setup of sediment assay (I) over 5 day exposure period (a). Free swimming juvenile zebrafish (20–25 dpf) foraging on particulate matter in upper-most sediment layer (b). Arrow indicates ingested sediment accumulated in the gut of fish (body length, 7.8 mm) at the end of exposure (c).

4.4.4 Impact of sediment-borne EE2 on transcript levels of *cyp19a1b* and *vtg1*

To explore the applicability of the *20dpfZF* as a free-swimming independent experimental model, exposure to EE2 contaminated sediment via water was performed (Fig. 16) (I), in according to the regulatory requirements that apply to the use of adult animals in toxicological research. Commercial salts e.g., Reef Crystal, Instant Ocean etc., are routinely added to system water for optimal growth of zebrafish in laboratory conditions (Westerfield 2007). The role of salt addition was investigated to explore any influence of salt concentration, albeit at a low range as used in this study, on desorption of sediment-bound EE2, possibly affecting bioavailability and the corresponding estrogenic response in exposed zebrafish. One-way ANOVA analysis of normalized gene expression showed significant induction of both *cyp19a1b* (F (4, 10) = 1096.501, $p < 0.001$) and *vtg1* (F (4, 10) = 2471.846, $p < 0.001$) of *20dpfZF* when exposed to sediment

spiked with nominal 3 µg EE2 g^{-1} dw over three to five days (Fig. 17a, b). Between the exposures (Sed 1 and Sed 2) conducted over three and five days, Tukey's HSD *post-hoc* test showed that fish exposed to sediments with EE2 exhibited higher induction of *cyp19a1b* than from water-borne exposure (nominal 150 ng EE2 l^{-1}) at 63- and 50-fold change, respectively (Fig. 17a). Further up-regulation in Sed 2 fish, with a 74-fold change, compared to EE2-water exposure, indicated the influence of the extended exposure duration.

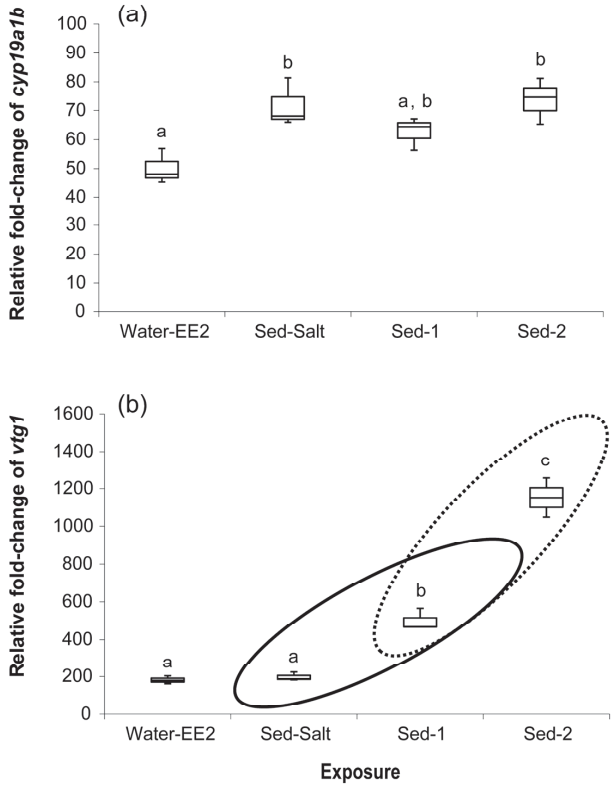


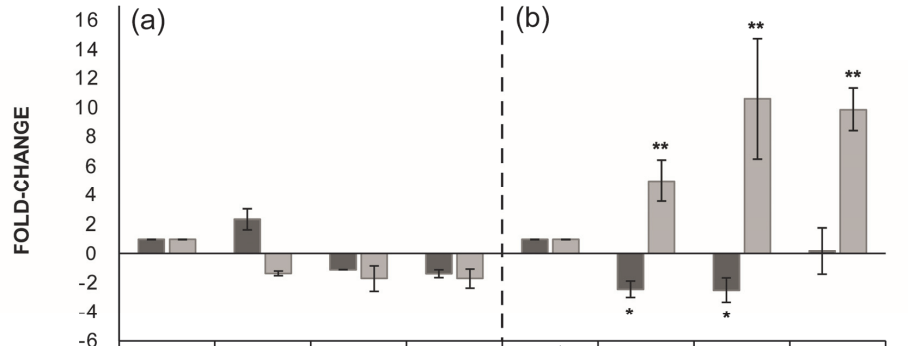
FIGURE 17 Gene induction in *20dpfZF* caused by sediment-borne EE2 (I). Fold change of *cyp19a1b* (a) and *vtg1* (b) from whole body homogenate of early juvenile zebrafish (23–25 dpf) exposed to sediment spiked with EE2. Fish (n = 3 pools) were sampled at the end of 3 d for Ref-Sed (PJ, Lake Palosjärvi), Water-EE2 (150 ng l^{-1}), Sed 1 + Salt and Sed 1. Sed-2 exposed fish were sampled at the end of 5 d. Box-plots represent normalized gene expression expressed as fold-change relative to control or reference exposure (Ref-Sed). Values are mean ± SD (15 fish per triplicate pool). Means with different letters are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's HSD *post-hoc* test. Note solid loop represents effect of salt concentration, and dotted loop represents effect of exposure duration.

The 3d exposures (Sed 1 + Salt, Sed 1), which differed only in respect of the electrolyte content, showed higher induction (72-fold) of *cyp19a1b* compared to

that of Sed 1, though it was not significantly different (Tukey's HSD, $p = 0.473$). The expression of *vtg1* showed very pronounced up-regulation patterns, increasing from EE2 in water (181-fold) to Sed 1 (500-fold) and Sed 2 (1152-fold) (Fig. 17b). Unlike *cyp19a1b*, *vtg1* showed significantly lower induction in Sed 1 + Salt (198-fold) when compared to Sed 1 (500-fold), possibly as an indication of the difference in the fraction of EE2 taken up by the fish (Tukey's HSD, $p < 0.001$). The *20dpfZF* life-stage was thus found to be practical and responsive to different media, including here the exposure to EE2-spiked sediment as a free-accessible contact assay. Overall, the *20dpfZF* exposed to sedimental EE2 via water highlighted the role of salt concentration as well as the duration of exposure in the induction of estrogen-responsive genes, *cyp19a1b* and *vtg1* (Fig. 17 a, b) (I).

4.4.5 Impact of sediment-borne OTCs on transcript levels of *cyp19a1b* and *vtg1*

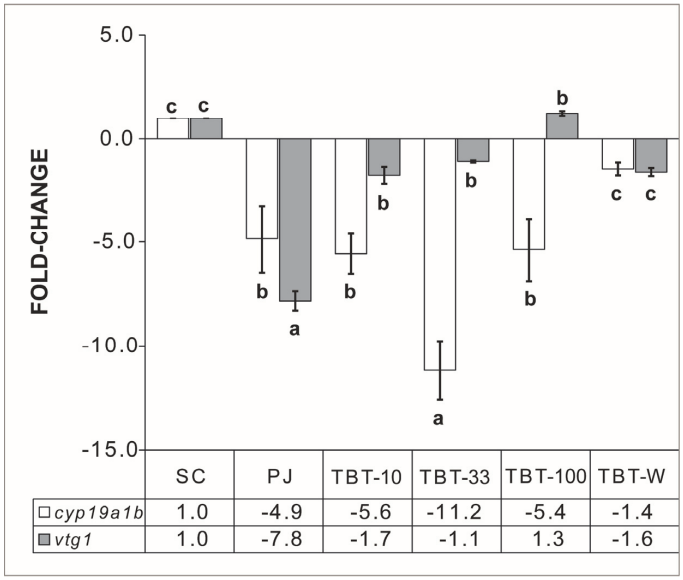
To investigate transcriptional alteration of estrogenic molecular biomarkers, *cyp19a1b* and *vtg1* in *20dpfZF* by exposure to naturally contaminated (HL) and sediment spiked with TBT (Fig. 18), early juvenile zebrafish were exposed to contaminated sediment via water (III) in experimental units depicted in Fig. 10.



	PJ[a]	HL-10	HL-33	HL-100	PJ[b]	TBT-10	TBT-33	TBT-100
cyp19a1b	1.0	2.3	-1.1	-1.4	1.0	-2.4	-2.5	0.2
vtg1	1.0	-1.4	-1.7	-1.7	1.0	5.0	10.6	9.9

FIGURE 18 Fold-change (± SD) of *cyp19a1b* and *vtg1* transcript levels in *20dpfZF* used in sediment assay *in vivo* (III). Experimental units ($n = 3$, each pooled of 15 animals) exposed for 3 d to dilution series of (A) industrial sediment sampled from Lake Huruslahti (HL), Finland, and (B) reference sediment (PJ) spiked with TBT. The combined results of the OTC-analysis of sediments showed that PJ, HL-10, HL-100, TBT-10 and TBT-100 contained 0, 5, 67, 185 and 1620 µg TBT kg^{-1} dw, respectively. From measured concentrations of TBT, HL-33 and TBT-33 contained 22 and 540 µg TBT kg^{-1} dw nominally, to represent an overall dilution series from 5 up to 1620 µg TBT kg^{-1} dw PJ, uncontaminated sediment control; HL-10, HL sediment nominal concentration 10 % of HL-100; HL-33, 33 % of TBT-100. PJ[a] and PJ[b] indicate sediment controls for HL- and TBT-spiked sediment assays, respectively. Asterisks indicating significant differences are shown as * ($p < 0.05$), ** ($p < 0.001$) as determined by one-way ANOVA followed by Tukey's HSD *post-hoc* test.

When compared to the PJ background, being alike in all sediment assays using *20dpfZF*, there was statistical significance between transcript abundance in exposure groups compared to the reference group (PJ) as determined by one-way ANOVA for *cyp19a1b* (F (3, 8) = 8.731, p = 0.007) and *vtg1* (F (3, 8) = 50.067, p < 0.001) respectively (Fig. 18b). Tukey's HSD *post-hoc* test showed statistically significant down-regulation of *cyp19a1b* by TBT-spiked (TBT-10 and TBT-33) sediments in comparison with the control (p < 0.05) (Fig. 18b). Unlike HL-sediment assay (Fig. 18a), *20dpfZF* exposed to TBT-spiked sediment showed statistically significant down- and up-regulation of *cyp19a1b* and *vtg1*, respectively (Fig. 18b). Water-borne exposures to TBT and EE2 did not cause statistically significant changes of *cyp19a1b* (p > 0.05) compared to the solvent control, though respective 2-fold average down- and up-regulations were noted (Fig. 19). However, *vtg1* showed pronounced up-regulation (24-fold, p < 0.05) when exposed to EE2 in water.



	SC	PJ	TBT-10	TBT-33	TBT-100	TBT-W
cyp19a1b	1.0	-4.9	-5.6	-11.2	-5.4	-1.4
vtg1	1.0	-7.8	-1.7	-1.1	1.3	-1.6

FIGURE 19 Fold-change (± SD) of *cyp19a1b* and *vtg1* in *20dpfZF* (n = 3 pooled samples of 15 each) exposed for 3 d to dilution series of industrial sediment sampled from Lake Huruslahti, Finland (III). HL-100 contained 67 µg TBT kg^{-1} dw. SC, solvent control; HL-10, HL sediment nominal concentration dilution to 10 % of HL-100; HL-33, 33 % of TBT-100; TBT-W, water-borne TBT 100 ng l^{-1}; EE2-W, water-borne EE2 30 ng l^{-1}. Different letters along bars represent significant differences in transcriptional fold-change of *cyp19a1b* (one-way ANOVA; p < 0.05) and *vtg1* (Welch ANOVA; p < 0.05) among exposure groups.

4.4.6 Impact of sediment contaminated by the pulp and paper industry on *cyp19a1b* transcript levels

To assess the impact of pulp and paper mill contaminated sediments on estrogen-responsive biomarkers in early juvenile zebrafish *20dpfZF*, animals were exposed to sediment core samples from the vicinity of a mill (IV). In order to compare recent and past pollution of benthic habitat, surface (0–5 cm) and subsurface (20–30 cm) sediment layers were probed by *20dpfZF* assay (Fig. 20).

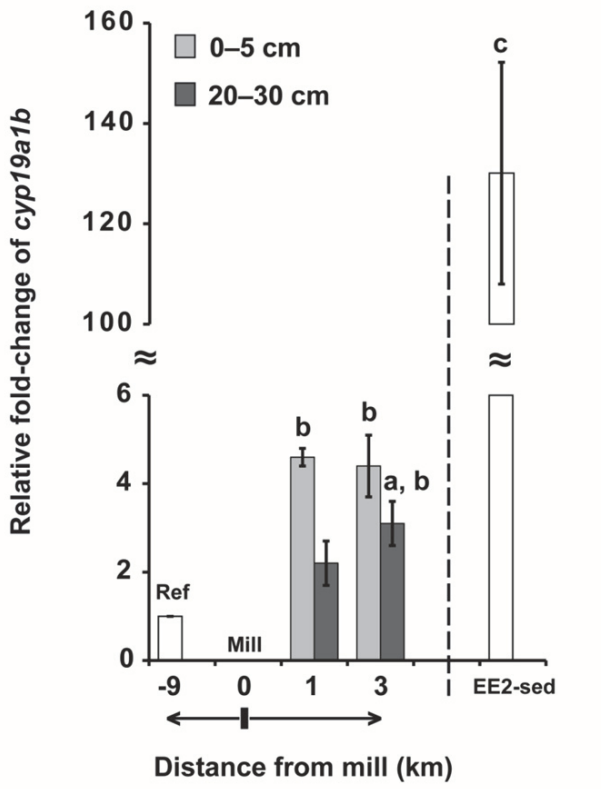


FIGURE 20 Changes in *cyp19a1b* mRNA levels in *20dpfZF* (n = 15) exposed to sediment from sites in vicinity of the pulp and paper mill in Southern Lake Saimaa (IV), Finland. Reference sediment (0–10 cm) was collected from Lake Rautniemi, 9 km upstream from the mill. Upper (0–5 cm) and lower (20–30 cm) sediment layers represent recent and more historical pollution at sites downstream from the mill. Positive control, EE2-sed is sediment spiked with 17α-ethynylestradiol (EE2) with concentration 3 µg EE2 kg^{-1} dw. Bars represent normalized mRNA level (± SD) relative to reference sediment. Different letters above bars represent significant difference between exposures ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's HSD *post-hoc* test.

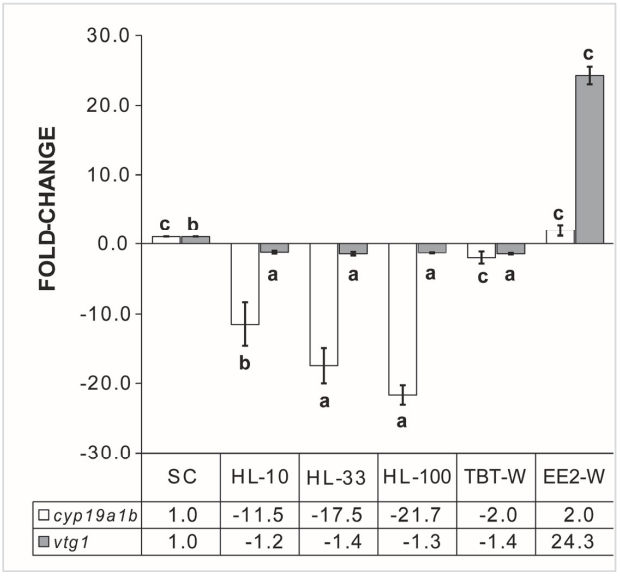
In the *cyp19a1b* transcript response, when compared to sediment from the upstream reference site (RN), exposure to both downstream areas from the pulp and paper mill (1, 3 km) induced significant up-regulation (though non-significant by 1 km at 20–30 cm) compared to the control group (RN) as one-way ANOVA for *cyp19a1b* indicated F (6, 14) = 544.590, $p < 0.001$ (Fig. 20). Tukey's HSD *post-hoc* test revealed significant induction of *cyp19a1b* between 1 km downstream sediment exposures to the surface (0–5 cm; 4.6-fold) and deep (20–30 cm; 2.2-fold, $p = 0.001$), thereby indicating higher estrogenic potency in recently deposited sediment. This pattern was also obvious at the 3 km site, the 0–5 cm sediment layer however not showing statistically significantly difference in *20dpfZF* exposed to 20–30 cm layer ($p = 0.174$). In confirmation of *cyp19a1b* response to estrogenic exposure, a separate EE2-spiked sediment induced up-regulation of *cyp19a1b*, albeit at a pronounced level (130-fold; $p < 0.001$, Fig. 20). Unexpectedly, our results did not show statistically significant induction of *vtg1* in juvenile zebrafish ($p > 0.05$) assayed with downstream SLS sediment (IV). Again, the positive control using EE2-spiked sediment was as predicted (I) and revealed an 8-fold increase of *vtg1* in exposed fish relative to the upstream reference RN ($p = 0.01$) (IV).

In summary, the up-regulation of *cyp19a1b* by exposure to the upper layers of sediment clearly highlights the estrogen-like impact of compounds deposited during the last decade from effluents discharged from the pulp and paper mill.

4.4.7 Role of reference sediment

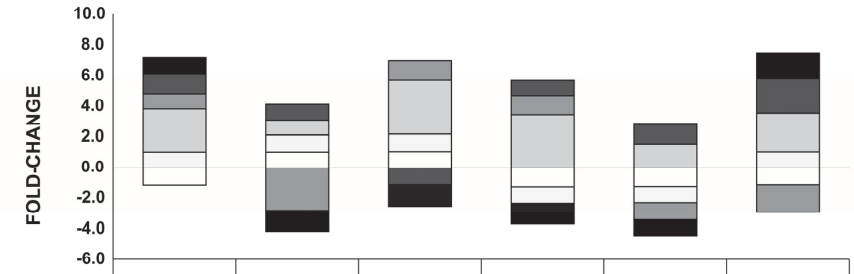
Besides being used as reference sediment, the pristine lake sediment PJ, when compared with water control (SC), showed down-regulation of both *cyp19a1b* (5-fold) and *vtg1* (8-fold) in *20dpfZF*, i.e. revealing an overall anti-estrogenic potential (Fig. 21) (III).

At the southern Lake Saimaa, the upstream site (RN) was selected as a reference to compare with the contamination of the downstream sites from the mill (IV). When taken as reference site, the PJ-based fold-change revealed no significant induction of *cyp19a1b* at both downstream sites (Fig. 22). Thus, for the whole sediment assay with *20dpfZF*, selection of reference sediment appeared as an important methodological issue. When fold-signals in gene transcripts are small, although statistically significant, a risk of false positive or false negative genomic outcome may arise.



	SC	HL-10	HL-33	HL-100	TBT-W	EE2-W
cyp19a1b	1.0	-11.5	-17.5	-21.7	-2.0	2.0
vtg1	1.0	-1.2	-1.4	-1.3	-1.4	24.3

FIGURE 21 Fold-change (± SD) of *cyp19a1b* and *vtg1* in *20dpfZF* (n = 3 pooled samples of 15 each) exposed for 3 d to dilution series of PJ sediment spiked with TBT (III). TBT-100 represents 1620 µg TBT kg^{-1} dw. SC, solvent control; PJ, Palosjärvi; TBT-10, TBT-spiked nominal concentration 10 % of TBT-100; TBT-33, 33 % of TBT-100; TBT-W, water-borne TBT 100 ng l^{-1}. Different letters along bars represent significant differences in transcriptional fold-change of *cyp19a1b* and *vtg1* among exposure groups (one-way ANOVA; $p < 0.05$).



	PJ	RN	1km 0-5	3km 0-5	1km 20-30	3km 20-30
cyp1a-PJ	1.0	-1.3	-1.5	-1.3	-1.1	**1.7**
cyp1a-RN	1.3	1.0	-1.1	1.0	1.2	**2.2**
cyp19a1b-PJ	**1.0**	**-2.8**	**1.2**	**1.2**	-1.1	**-1.8**
cyp19a1b-RN	**2.8**	**1.0**	**3.5**	**3.5**	1.5	**2.6**
vtg1-PJ	1.0	1.1	1.2	-1.1	-1.1	1.0
vtg1-RN	-1.1	1.0	1.0	-1.2	-1.2	-1.1

FIGURE 22 Comparison of fold-changes of *cyp1a*, *cyp19a1b* and *vtg1* in *20dpfZF* exposed to SLS sediment (IV), assessed relative to two separate reference sediments (RN, Lake Rautniemi and PJ, Lake Palosjärvi (reconstituted sediment)). For the details of groups, see the legend of Fig. 20. Fold-changes considered for study IV was calculated relative to RN as the reference site, being the authentic upstream site from the pulp and paper mill. Note that the statistically significant differences in fold-change of *cyp1a* and *cyp19a1b* are shown in bold.

5 DISCUSSION

5.1 Ecotoxicological realism and relevancy of ELS assay tools

The primary finding of this research is that a 20 d old zebrafish *20dpfZF* is a universally suitable ecotoxicological model, which may be utilised in the intelligent screening of environmental effects of local samples, mixtures like effluents, and single chemicals, with endocrine properties to alter regulation of physiological steroids. This stage can be compared with adult and embryonic studies (Muncke and Eggen 2006, Sawyer *et al.* 2006) which have been carried out under the advanced regulatory requirements for experimentation of aquatic toxicities (OECD 2012), with specific current emphasis on the use of fish ELS (OECD TG 236). Although adult fish have been more routinely used in studies of comparative toxicology, the early-juvenile life-stage considered in this work was more advantageous for the ecotoxicological experimentation with the possibility of large sample sizes.

The life-stage of zebrafish investigated can be described as early juvenile, because at the time of 20 dpf they possess initial ovary-like tissue that transits into sex-specific gonad phenotypes. Changes during such a labile phase (3 to 6 weeks post fertilization; wpf) of gonadogenesis (Takahashi 1977, Örn *et al.* 2003, Maack and Segner 2004) are detected by estrogen-responsive gene expression. Results from studies included in this thesis can be broadly discussed as a response to xenoestrogens as single chemicals (I), mixture combinations (II), and habitat-contamination (III, IV).

Robust gene level responses (I, III, IV) including estrogenic and the other steroidogenic ones, are suggested to give a mechanistic understanding on impacts of environmental chemicals, their dynamics of effects and, most importantly, to provide extrapolations which may be used in environmental risk assessment. With estrogen-responsive genes (*cyp19a1b* and *vtg1*) as indicative biomarkers of estrogenicity, this study also showed that mixture effects (II) at the molecular level are functional in *20dpfZF*. Changes in transcription of ER-mediated (I–IV) and AhR-mediated (II, IV) biomarkers used

in my studies highlight the modulating potencies of environmental chemicals and samples. Introduction and advancing of the *20dpfZF* satisfied several scientific, practical, and cost-effective requirements, though the main focus of this study was to select and evaluate a critically sensitive and ecologically defensible life-stage as a suitable screening model for xenoestrogenicity. Importantly, being self-controlled with respect to feeding and spatial behaviour, this life-stage *20dpfZF* monitors its habitat widely, as observed even in sediment exposures (I, III, IV). The information regarding the gene-level response determined here in water-based chemicals and exposures could be primarily compared to a sediment exposure carrying a potent xenoestrogen with a substantial trait of hydrophobicity, EE2 (log K_{ow} 4.15) (Lai *et al.* 2000).

The integration of the 3Rs (replacement, refinement, reduction) principles in animal testing regulation has resulted in the increased use of lower vertebrates, such as the zebrafish *in vivo* (ELS bioassays) and *in vitro* (tissue samples) and other non-invasive technique-based studies. In part, this has broadened our understanding of biological responses in development (Hill *et al.* 2005), endocrine disruption (Segner *et al.* 2003, Van den Belt *et al.* 2003, Jin *et al.* 2009) and reproduction (Brion *et al.* 2004) among other effects. Despite the evident applicability of the very early juvenile developmental stages, the question of the suitability of zebrafish arose since they are a non-native species. However they are able to perform as an ecotoxicological model for diverse locations in nature, including for example as laboratory tools for boreal waters. Besides, it still is cost-effective to rear the fish until 2–3 week old (*20dpfZF*), with comparable maintenance effort with zebrafish ELS, e.g. 5 dph. Here it is suggested that the relevancy of zebrafish ought to be focused on the *20dpfZF* being more bio-functionally diverse than very early stages of embryos, as an assay tool for ecotoxicity.

5.2 Some methodological perspectives of the current study

This study introduced a time window (20–25 dpf) that was suitable, also in terms of stably expressed reference genes (beta-actin, *β-actin*; elongation factor 1a, *ef1a*), considering the significance of reliable reference genes required to normalize target gene expressions (Vandesompele *et al.* 2002). Supporting this fact, even earlier embryonic stages (48–120 hpf in Muncke and Eggen 2006; 1–120 hpf in Sawyer *et al.* 2006) with steady expression of *β-actin* were considered to be suitable reference for mRNA quantification of other genomic signals. In addition to estrogen-responsive genes, *cyp1a* transcription was found to be functional in *20dpfZF* (II–IV), as shown in embryonic ELS as well (Jones *et al.* 2010).

Very early life-stages of zebrafish have become popular in the assessment of a variety of environmental factors in recent years (Nagel 2002, Braunbeck *et al.* 2005, Scholz *et al.* 2008), including the assessment of toxicity of environmental chemicals (Incardona *et al.* 2004, McClain *et al.* 2012) and

contaminated sediments (Hollert *et al.* 2003, Wu *et al.* 2009). The *20dpfZF* instead is an actively foraging life-stage (Westphal and O'Malley 2013), still widely ecological alternative for chemical fate-based experimentation, and an authentic fish species in subtropics. While the fish early life-stage toxicity test required experimentation to end prior to 30 dph (OECD TG 210), zebrafish between 20–25 dpf (dph = 3 + dpf) age during experimentation were effectively responsive to estrogenic signals.

The selection of appropriate controls is a basic requirement for the quantification of reliable and consistent responses in early juvenile zebrafish. The choice of a positive control was obvious, with EE2 a benchmark of estrogenic potency in water as well as sediment exposures. To assess sediments for EDCs at industrially contaminated areas, unknown interactions are possible to be investigated with inclusion of reference compounds belonging to a range of agonistic or antagonistic influence on estrogenicity. In my studies, the selection of clean controls depended on the exposure sample used for the respective series; contaminated water and sediment compared to solvent control (0.001 % MeOH) and reference sediment, respectively. Furthermore, specific to the requirements of each of the sediment assays (I, III, IV), two separate sediments, Lake Palosjärvi PJ (I, III) and Lake Rautaniemi RN (IV), were respectively used as blank references. Specific to our studies (III), both *cyp19a1b* and *vtg1* were down-regulated in *20dpfZF* animals exposed to the PJ (reference site) (p = 0.001), when compared to solvent control. The difference in either transcriptional response emerged with comparison to PJ, as the control (III). The sampling site for the reference sediment was located at the bay of Myllylahti at the Lake of Palosjärvi (PJ), in Toivakka municipality, Central Finland. Though the lake has been an unindustrialised area and basically pristine by edaphic background, apparently, unknown natural anti-estrogenic factors may be involved in the PJ sediment. It is common knowledge that plants are sources of estrogenic compounds, mainly belonging to chemical classes such as isoflavones (genistein, diadzein), lignans (pinoresinol) and coumestans (coumestrol), which may even have anti-estrogenic impact (Horn-Ross *et al.* 2003), some even important pharmaceuticals in use today, like taxol (Adams *et al.* 1993), resveratrol (Asensi *et al.* 2002), genistein (Behloul and Wu 2013), among others (Usui 2006, Liu *et al.* 2010). It may therefore be suggested that even clean sediments formed to a large extent from plant-origin material can have some potency of similar nature.

A partial survey of aquatic plants in bay Myllylahti of PJ made in September 2013 (Oikari, A., personal communication), identified as domimant macrophytes (the lake reed, lake bulrush and yellow water-lily) with other macrophytes (the lake horse-tail and lake butter-cup). While no chemical surveys of these plants and adjacent sediment is available yet, the influence of factors other than those discussed in Studies III and IV, more extensive chemical analysis might reveal compounds possibly contributing to the anti-estrogenic effect observed for that site. A further suggestion also arises in whole sediment assays in general, including those by *20dpfZF* that future studies

ought to include two or three clean reference sediments in experimental series. Finally, a wider survey of pristine and upstream sites in boreal waters is recommended here, to constitute a national or regional database of reference samples (sites).

Another important issue regarding to 3R principles in experimental animal studies, emerging from this thesis, is the ethical relevance of low sublethal exposure (III, IV). We strongly suggest the acceptance of advanced life-stages, e.g. early juvenile fish, in sublethal toxicity studies. A wide set of sublethal gene responses guarantee minimal pain experience to the animal compared to lethal levels of chemical exposure. In nature, as chemical exposure takes place also by permit (ISO 14001:2004), natural populations of aquatic organisms are similarly and continuously exposed to suggested "clean" media with sub-threshold contamination levels.

5.3 Responses of *20dpfZF* to xenoestrogenic model compounds (I, II, III)

5.3.1 Background information

Internationally, the most universal guideline to assess environmental properties of each commercial chemical is that of OECD, with novel amendments being introduced to working packages to accommodate the increasing concern from environmental chemicals (OECD 2014). For assessment of hormonally active substances, these guidelines are followed with more or less minor modifications. The extrapolation of chemical-mediated effects from the organism to the population level depends on the extent of sex differentiation in species with dimorphic phenotypes such as occur in zebrafish. In such species, the regulation of reproduction and associated endocrine processes are manipulated by feedback from endogenous steroid production and environmental factors together, including photoperiod, temperature, and chemical cues (Hill *et al.* 2005). Gene transcription of key biomarkers associated with pathways of the hypothalamus-pitutary-gonadal (HPG) axis presents strong phenotypic anchoring of a hazardous chemical assault on teleostean species (Klaper *et al.* 2006, Santos *et al.* 2007).

5.3.2 Alteration of steroidogenic pathway genes

The conversion of cholesterol into active sex hormones such as estradiol and testosterone occurs mainly in the brain, kidney and gonads, via steroid biosynthetic pathways involving multiple enzymes (Fig. 1, Payne and Hales 2004). According to results presented here (I), *star* transcript level in *20dpfZF* showed no major change to EE2 as did *cyp11a1* and *hsd3b1* genes, which encode enzymes that catalyse the first two steps of the steroidogenic pathway, displaying overall a lesser relative change. The expression of *cyp11a1*, also

named P450scc, which encodes for the cholesterol side-chain cleavage enzyme, was minimally expressed during the larval stage until independent expression when approaching sexual maturity (30 dpf; Hu *et al.* 2004). Lower transcript levels of *star*, *cyp11a1*, and *hsd3b1* in zebrafish exposed to EE2 (I), a potent estrogenic agonist, suggest that diverse regulatory processes are taking place. The male fathead minnow, another small-bodied model teleost, also showed down-regulation of Star mRNA when exposed to 10 ng EE2 l^{-1} (Filby *et al.* 2007). Interestingly, the lack of corresponding alteration of steroidogenic (Star and Cyp11a1 mRNA) and biotransformation (Cyp1a mRNA) responses has been demonstrated in adult zebrafish exposed to water-soluble fraction of crude oil (Arukwe *et al.* 2008). In *20dpfZF*, the only consistent steroidogenic signal was that of aromatase (*cyp19a1b)*.

To conclude, although transcriptional response as shown by *star*, *cyp11a1* and *hsd3b1* did not suggest steroidogenic alteration in *20dpfZF* exposed via water, though *cyp19a1b* showed a clear dose-related up-regulation pattern (I).

5.3.3 Aromatase and vitellogenin transcription is affected by xenoestrogens

The choice of *cyp19a1b* as a core endpoint for the life-stage *20dpfZF* was determined from concentration-specific transcript abundance compared to other steroidogenic enzymes (*star*, *cyp11a1* and *hsd3b1*; I). The presence of estrogen response element (ERE) in the promoter region of *cyp19a1b*, but absent on *cyp19a1a*, has been shown to explain the estrogen-responsiveness of *cyp19a1b* (Callard *et al.* 2001, Kazeto *et al.* 2001, Diotel *et al.* 2010). Thus, being a potential biomarker of estrogenicity, interestingly, the *cyp19a1b* signal has also been employed in transgenic zebrafish specifically as an estrogenic-sensitive screen (Tong *et al.* 2009, Brion *et al.* 2012). However, in point of fact *in vitro* gene constructs lack *in vivo* interactions that are crucial to inherently establish an authentic causal effect.

Because *cyp19a1b* has a key role in steroid biosynthesis at the life stage of *20dpfZF*, being involved in the conversion of androgen to estrogen, it qualified as a compelling signature of xenoestrogen-induced effects. Considering the role of gene expression on phenotypic sex determination, it allows a comparative understanding of chemicals (agonists) with similar MoAs. Taken together with maximal expression of Vtg from 5 d exposure in zebrafish embryos (0–5 dpf) (Muncke and Eggen 2006), the mRNA detection of the altered *vtg1* response for our study was a practical choice. In water-borne exposures (I, II), the induction of *vtg1*, as a response to estrogen-mediated cellular responses in the (neuro-) steroidogenic pathway, was consistent with the downstream outcome of aromatase activity (Fig. 1).

In Study III, assayed by *20dpfZF*, the response of the water-borne TBT pure chemical showed the lowest anti-estrogenic effect, when treated in a technically similar way to the sediment exposures. Results from Study IV, however, showed clear induction of *cyp19a1b* and not of *vtg1*, revealing the sensitivity of aromatase response to a low level exposure to some pure

chemicals and environmental sample. In all, the *20dpfZF* along with its sensitivity to estrogenic exposure was found to be a defendable assay tool.

5.4 Responses of *20dpfZF* to xenoestrogenic mixtures (II)

There is an international harmonization on evaluation of toxicity by effluent emissions, i.e. mixtures, containing follow-up strategies, by legal permit. Monitoring of effluent toxicity and other properties, like priority pollutants and inorganic plant nutrients, facilitates implementation of e.g. European water policy. The current list of priority substances in European water management given by WFD (COM (2011) 876), include pharmaceuticals (EE2 and diclofenac), alkylphenols (nonylphenols and octylphenols), metals (compounds of lead and mercury), tributyltin compounds, among others. They are potentially harmful to aquatic biota, but are yet to be fully approved considering the financial costs of regulatory implementation. Voluntary environmental programs developed by the International Organization of Standardization (ISO 14001:2004) provide practice guidelines to polluting organizations by regulating environmental emissions.

This far rather few other studies have examined gene induction as an endpoint of joint toxicity of chemical mixtures either *in vitro* (Staal *et al.* 2007, Gräns *et al.* 2010) or *in vivo* (Sun *et al.* 2009, Johns *et al.* 2011). In our studies with *20dpfZF*, mRNA abundance of *cyp19a1b*, *esr1* and *vtg1* in mixture (BPA + NP + OP) exposures suggested estrogenic effects of the mixture combinations (II). Additionally, it also suggested limited influence or no influence at all of xenoestrogen metabolism on the estrogenic effects of the mixture combinations. The formulation of equitoxic mixtures comprised components each representing a toxic unit, EC50 or NOEL (of individual threshold response), where individual components even in a simple mixture may compete for the binding sites (Fig. 2), based on the localized concentration as well as the binding potential and related structural facilitation (Koda *et al.* 2002). For a mixture of similarly acting chemicals, the combined kinetics of binding and displacement of individual agonistic components to the steroid receptor is therefore a vital trait for eliciting adverse alterations. As the toxicity outcome of combined action by a mixture depends on the properties of individual chemicals, besides promoting statistical additivity, it can also include sub- and super-additive interactions (Kortenkamp and Altenburger 1998, Rajapakse *et al.* 2001). The fixed-ratio exposure design indicated a potentiation of low-level *vtg1* activation (2.5-fold) in Study II. It was thus evident that of the two key biomarkers, *vtg1* proved a robust indicator of mixture-induced effects. This goes along with studies using juvenile rainbow trout (Thorpe *et al.* 2001), adult and embryonic fathead minnow (Brian *et al.* 2005, Johns *et al.* 2011) and juvenile zebrafish (Lin and Janz 2006). Theoretically, the joint effect of BPA + NP + OP (II) resulted in minimum additive response of the component chemicals.

The down-regulation of *cyp1a* transcripts could be the combined estrogenic effect of the mixture of BPA + NP + OP (II), also evident from sediment-borne EE2 (IV), which may be due to the inhibitory impact of EE2 (Hasselberg *et al.* 2005). Although the experimental fish in our studies were just early juveniles and not fully differentiated, the pronounced aromatase induction, central to E2 production, supports the hypothesis of indirect estrogenic impact. However, estrogenic chemicals have also been shown to impact estrogen metabolism. Specific to the 20-dpf juveniles used in my study, other studies have shown peak basal expression of Cyp1a around 20-dpf (Jönsson *et al.* 2007). Relative to the basal expression, a decrease of such supplementary estrogen metabolisers can be another indicator of disrupted endocrinic metabolism. Moreover, the decreased activity of CYP1A, perhaps as an adaptive response allows increased cellular and tissue hormone levels in maturing fish (Forlin and Hansson 1982, Arukwe and Goksøyr 1997).

Xenoestrogenic exposure may also cause increased concentration of active estrogens by impairment of estrogen metabolizing enzymes, i.e. sulfotransferases (Kirk *et al.* 2003). On the basis of our results, while it is difficult to identify any single explanation, the transcriptional response of *cyp1a*, being inversely associated with that of *vtg1*, is in accordance with earlier reports (Gräns *et al.* 2010), suggesting "cross talk" between AhR and ER-signaling pathways. To explore the utility of this life-stage (*20dpfZF*) in a more elaborate manner, however, further investigations are required, not limited to estrogenic or anti-EDCs.

5.5 Responses of *20dpfZF* to freshwater environmental samples (III, IV)

5.5.1 Background information

The EU Water Framework Directive 2000/60/EC (EU 2000), enacted in 2000, was revised in 2008 (Directive 2008/105/EC, EU 2008b) to include environmental quality standard limits for the regulation of 33 priority substances and 8 priority hazardous substances in polluted surface waters, sediments and biota. In that regard, the focus of national implementation programmes of Baltic Sea countries is also directed to the control of hazardous substances in the Baltic catchment area (HELCOM 2009, 2013). Implementation of the HELCOM Baltic Sea Action Plan (BSAP) includes assessment of chemicals in sediment, soil and water resources. Further, to manage emissions from point sources, regulatory mechanisms (ISO 14001:2004), national programmes e.g. Finnish river basin management plans, are actively invested for emissions, for instance by whole effluent assessment. Besides effluent quality, the sediment, being central to the fate of organic contaminants, is a natural sink or source for many hazardous substances. In short the initiatives

are aimed to extend the relevance of sediment-related issues in policy making with help of the sediment quality triad (Chapman 1996), for management of European aquatic systems e.g. the European Sediment Research Network (SedNet).

Particularly with the management of organotin contamination, following an EU restriction on the use of TBT-based antifouling paints (IMO 2001), the role of sediments as depositories of OTCs is obvious. Although remediation activities are adopted to facilitate a healthy recovery of the contaminated sites, tributyltin compounds identified as one of the priority hazardous substances, are highly hydrophobic. Besides chemical monitoring, evaluation of remediated sites should also include biological endpoints, to confirm the actual status of recovery.

Therefore, a universal fish model would serve more ecologically defensive instrument to assess hazardous chemicals. In my studies, we discussed the xenoestrogenic impact of two industrially contaminated sites, i.e. from deposition of OTCs (III), and pulp and paper production (IV) residues. Zebrafish (*20dpfZF*), as a freshwater species, served to highlight the toxicological implications of industrial contamination on the endocrine status of early juvenile ELS (III, IV). Whole sediment bioassays mimicked real-world exposure scenario, with the possibility of evaluating sublethal toxicities, in particular steroidal endocrine disturbances. Moreover, the sediment exposure presented at least similar, if not more, effective uptake compared to the pure chemical by water, with sediment acting as a continuous source. While both *cyp19a1b* and *vtg1* are, biomarkers to xenoestrogenic impact (I, II), the type of contamination in studies III and IV contrasted variation in these transcripts, despite both mediated by the ER pathway.

5.5.2 Industrial sediment OTC: responses of estrogenic biomarkers

Although diverse animal taxa have been shown to be responsive to OTC accessibility (Horiguchi 2012, Sousa *et al.* 2014) via covalent and non-covalent interactions, toxicity to fish at target sites include membranes (Pavlikova *et al.* 2010), intracellular organelles like the mitochondria, DNA, nuclear receptors (Colliar *et al.* 2011, Grün 2012, Zhang *et al.* 2013), and specific endocrine effects. Key mechanisms of action appear to be caused by altered OTC-metabolism (Mortensen and Arukwe 2009) and inhibition of certain CYP enzymes (Cheshenko *et al.* 2008, Meyer *et al.* 2012). Traditionally, TBT and other OTCs are considered aromatase inhibitors, eventually acting on gonadal development and reproductive performance. Chronic exposure to as low as 1 ng TBT l^{-1} in developing zebrafish (0–30 days post-hatch) caused a male-biased sex ratio in 3–5 month adults (McAllister and Kime 2003). The significant down-regulation of *cyp19a1b* (compared to *vtg1*), showing 2.5-fold change in TBT-10 and TBT-33, indicates its sensitivity as an endocrine biomarker. The sediment dilution also revealed a non-linear transcription pattern of *cyp19a1b* between TBT-33 (nominal 540 µg TBT kg^{-1} dw) and TBT-100 (1620 µg TBT kg^{-1} dw). The dilution TBT-33 was the most potent exposure compared to other two ($p < 0.05$), with

maximal regulation of *cyp19a1b* (2.5-fold down-regulation) and *vtg1* (10.6-fold up-regulation). We thus conclude that the simulation of HL-sediment by controlled addition of only one ingredient, TBT, was not fully valid from an environmental point of view. Such a discrepancy in the potency of the HL sediment could be due to the additive effect of co-contaminants such as mercury, PCBs, PAHs and PCDDs, also detected in Lake Huruslahti, albeit each one being present in negligible concentrations (III).

The first approximation on MoA of OTCs of Lake Huruslahti (HL) was that it was similar to TBT, the dominant OTC in contaminant site. The authentic sediment site contaminated by industrial activity, HL, and the artificially contaminated (TBT-spiked) sediment represented a range of TBT-exposures, to which *20dpfZF* showed gene-specific patterns of transcription (Fig. 18a, b). Reduced average transcription of both *cyp19a1b* and *vtg1* in *20dpfZF* by exposure to HL-sediment (except HL-10 of *cyp19a1b*) showed that the OTC-contaminated sediment caused an apparent anti-estrogenic response. Although the decreases in transcription of either gene in animals exposed to HL-sediment (Fig. 18a) was not significantly different ($p > 0.05$), the overall regulatory patterns of both genes are more evident when responses of both exposures (HL and TBT-spiked), albeit independent assays, are assessed as a gradient of TBT dose-response relationship (Fig. 18).

In the case of TBT-spiked sediments, OTC-mediated response in *20dpfZF* showed significant down-regulation of *cyp19a1b* in comparison with control (Fig. 18b). Unlike bioassay with *native* HL sediment (Fig. 18a), *20dpfZF* exposed to TBT-spiked sediment showed significant down-regulation of *cyp19a1b* corresponding to up-regulation of *vtg1* (Fig. 18b). Such transcriptional incongruence of *cyp19a1b* and *vtg1* suggested that the endocrine effect of TBT on whole fish were as such the summation of tissue-specific variation of androgenic (brain) and estrogenic (liver and gonads) effects, respectively (McGinnis and Crivello 2011). While positive control (EE2) in this series, as in others as well, revealed expected up-regulation of *vtg1*, at lower concentrations, up-regulation of *cyp19a1b* can however be more sensitive compared to *vtg1* (I). Obviously TBT ought not to be characterized as a typical estrogenic xenobiotic despite it increased *vtg1* expression together with material present in reference sediment.

Despite its serving as a solid experimental background, some unknown interactions may have modulated the combined outcome of the PJ-diluted HL and TBT-spiked sediments (see 5.2). Interestingly therefore, boreal lake sediments appear to contain sometimes anti-estrogenic chemical factors as natural background. Regarding the source of contaminated HL-sediment, it has been reported that ca. 200 µg TBT kg^{-1} dw extends over 30 km, locally at least but most probably occurring evenly (Anon 2009). As HL-100 roughly matched with that concentration, the genomic changes may occupy very wide areas of waters downstream from the city of Varkaus. Besides, on the genomic level at low sublethal concentrations, non-linear dose response behaviours appear to be

more common than the traditional endpoints representing higher integrative level, such as fecundity and lethality (Krasnov *et al.* 2007).

Santos *et al.* (2006) showed that co-exposure of TBT and EE2 induced an estrogenic or feminising effect in developing zebrafish, overriding the anti-estrogenic effect of TBT alone. In polluted areas co-contaminants thus include a myriad of interactions causing a complexity of steroidogenic regulation, necessitating a whole sediment direct assay of the contaminated site, e.g. by using *20dpfZF*. The study (III) further employed the use of zebrafish early embryos to assess the toxicity of sediment elutriates from HL, and determine the severity of the source. It is important to remember that other MoAs of TBT may as well have acted in concert with *cyp19* involvement. Because of paucity of such information, further documentation of their roles at HL area is essential.

5.5.3 Industrial sediment PPM: responses of estrogenic biomarkers

Effluents of pulp and paper industry have been extensively studied for their endocrine and reproductive impacts, similar studies with sediments being sparse however. In contrast to biomarker variation by OTCs in Study III, PPM-derived sediment contamination induced aromatase, but not vitellogenin, as the biomarker of estrogen exposure (IV). This result, though inconsistent with an ER-mediated sequence of events (Fig.1, Study I), extended the scope of *20dpfZF* as a probing tool for endocrine effects in the natural setting. Another interesting result of the study (IV) was the difference in the induction of responsive genes (*cyp19a1b*) in surface (0–5 cm; 4.6-fold) and deeper layers (20–30 cm; 2.2-fold), thereby indicating higher estrogenic potency in recently deposited sediment. Overall, the increased induction of *cyp19a1b* by exposure to the upper layers of SLS sediment highlights the estrogen-like factors deposited from effluents released from the pulp and paper mill.

The estrogenic influence of sediments from the 1 km site could be linked to single effector chemicals, for instance to the wood-derived β-sitosterol, a known estrogen-mimic in fish (MacLatchy and Van Der Kraak 1995, Mellanen *et al.* 1996, Tremblay and Van Der Kraak 1999, Nakari and Erkomaa 2003, Sharpe *et al.* 2007). Monitoring data of the Southern Lake Saimaa sites has revealed marked reduction in β-sitosterol concentration in the 1 and 3 km sediments (IV). Another potential hormonal agonist in SLS sediment can be betulinol, a triterpene alcohol occurring in the bark of logged trees in vicinity of the mill (Mellanen *et al.* 1996, Lahdelma and Oikari 2006). However, the induction of *cyp19a1b* cannot be solely attributed to the estrogenic potential of the SLS sediment. Binding affinities of competitive ligands can result in varied (anti-)estrogenic responses, as shown in the fish masculinization which was caused by exposure to androgenic contaminants, rather than by the inhibition of aromatase (Orlando *et al.* 2002). Örn *et al.* (2006) showed elevated Vtg protein concentration in juvenile zebrafish (10–38 dph) exposed to effluent from a totally chlorine-free (TCF) bleached kraft mill, and over longer exposure, the developing fish exhibited an androgenic response at 60 dph, attributed to the aromatisation of androgens in the mill effluent.

The steroidal content of wood and corresponding effluents from PPMs has been suggested as the source of xenohormones (Jenkins *et al.* 2003, Carson *et al.* 2008), a view that is not fully supported by efficient modification by secondary treatment systems (van den Heuvel 2010) which limit their release for active uptake by fish and aquatic organisms living downstream from PPMs. More definitive diagnosis is necessary to explain either argument. In study IV, all responses are indicative of the toxicity of the sediment that constituted the source samples, and not the effluent, although the extent of sediment contamination is influenced by effluent quality.

Although the up-regulation of *cyp19a1b* by SLS sediment, compared to no change in *vtg1*, was obvious, this result indicated estrogenic influence, even if at lower levels (IV). Given the various adverse outcomes of ER-mediated reduction in synthesis of Vtg in fish (ER-antagonism, aromatase inhibition and AR-agonism; Ankley *et al.* 2010), the induction of *cyp19a1b*, but not of *vtg1*, suggests competitive binding of steroid-receptors critical to reduced vitellogenesis. This may in turn provoke a feedback signal to increase estradiol production (by increased aromatase activity) to effectively compete with antagonist ligands to bind to ERs. A departure from simple estrogenic effects of PPM shown in other studies may additionally have resulted due to variation in species-specific sensitivity and experimental exposure design, but probably it is mainly due to the variable composition of effluent (Ellis *et al.* 2004). Although Mellanen *et al.* (1999) showed vitellogenin gene induction in field-exposed juvenile whitefish to PPM effluent from one of three mills in the Southern Lake Saimaa region, no similar estrogenic effect by Vtg-regulation was observed at the two other mill sites in that study.

Current effluent quality at SLS may differ from that reported in 2001 (IV), with for instance the concentration of another prevalent wood extractive, betulinol, in SLS sediment being as high as 3810 µg g^{-1} (dw) in the sediment depth of 20–25 cm, with surface sediment containing half of that concentration (Lahdelma and Oikari 2006). Theoretically, betulinol may potentially affect normal physiology and endocrine function in exposed fish (Christianson-Heiska *et al.* 2008) at SLS. And also, importantly, DHAA, being the most abundant RA in SLS, was recently reported to bind competitively to estrogen receptors, thereby eliciting an anti-estrogenic effect (Orrego *et al.* 2010). In contrast to Christianson-Heiska et al. (2008), Teresaki et al. (2009) showed that anti-estrogenicity of RAs such as DHAA is not accounted by competitive binding to ER. Additional information on the anti-estrogenic action of DHAA is suggested to occur indirectly through liver damage; the site for vitellogenin synthesis (Orrego *et al.* 2011). However, a more recent study opposed that claim, and suggested the disruption of estradiol metabolism by DHAA as an alternative explanation (Pandelides *et al.* 2014). Occurrence of androgens in effluents from bleached kraft mill (Larsson *et al.* 2006), and bleached sulphite mill (Parrott *et al.* 2003), could explain the potential for competitive binding of ER by AR-agonists, or even non-ER-mediated signalling, i.e. AR-based response, in exposed fish. Thus in the present study (IV) too, the significant

induction of *cyp19a1b*, but not *vtg1*, may be mediated by androgenic (Mouriec *et al.* 2009) or anti-estrogenic compounds from the lake sediment binding to estrogen receptors in developing zebrafish.

Transcription of *cyp1a* may be associated with sediment-derived retene contamination, as in the SLS region receiving discharges from the Kaukas pulp and paper mill (Soimasuo *et al.* 1998) and other studies (e.g. Werner *et al.* 2010). In our studies, sediments from sites 1 km and 3 km downstream of the mill failed to induce *cyp1a* in juvenile zebrafish, when compared to the upstream reference site (RN). Exposure to sedimental EE2 caused no transcriptional change in *cyp1a*, as was expected with the inhibitory effect of EE2 on fish CYP1A activity (Hasselberg *et al.* 2005), as is also shown in Study II. The induction of liver Cyp1a, generally measured as activity of 7-ethoxyresorufin deethylase (EROD), considered before as a distinct biomarker of impact by PPM effluent, has been decreasing with decreased emissions of AhR agonists from the mills. In SLS, for instance, the EROD response decreased by 90 % in the early 1990s due to discontinued chlorine-based bleaching of pulp (Oikari and Holmbom 1996). Later on, in 2004, short-term exposure of brown trout in the field of SLS revealed no significant difference in EROD between downstream and upstream sites in relation to the mill location (Oikari *et al.* 2010).

On the other hand, it was surprising that the deep sediment layers of 20–30 cm, representing a time before large technological transitions in 1992, revealed no increases in amounts of *cyp1a* transcripts by *20dpfZF* (IV), despite doing so in certain model AhR ligands (Billiard *et al.* 2002, Brinkworth *et al.* 2003, Hawliczek *et al.* 2012). We first suggested that the tentative effector, retene, had caused a pronounced induction of *cyp1a* transcription. Alternatively, the presence of unknown but bioavailable inhibitors of Cyp1a activity on EROD, such as bioflavonoids (Doostdar *et al.* 2000), may have been under action from the mixture of deposited compounds, originating in PPM effluents on the transcription of *cyp1a* in *20dpfZF*. Though, not significantly different, *cyp1a* showed higher induction when exposed to sediment depths (20–30 cm) at both downstream sites, representing the impact of remediation on the AhR-modulating potency of the recently deposited surface layers (0–5 cm). Actually, inhibition of mixed function oxygenase (MFO) activities has been occasionally observed, particularly due to untreated effluents of pulp from the paper industry (Ahokas *et al.* 1976). Perhaps also high TOC (ca. 11–15 % dw) of the SLS sediment (IV) decreased bioavailability of AhR-ligands (e.g. retene) with substantial hydrophobicity (log K_{ow} 6.4).

Overall, considering the sensitivity of *cyp19a1b* in water-borne exposure to XEs (I), results from sediment exposures (III, IV) further showed the impact of low-level toxicities on aromatase induction. In the absence of vitellogenin response to samples with low potency for induction, aromatase is a suitable candidate biomarker, whose sensitivity is not limited to estrogenic signals alone (Pagliarani *et al.* 2013). A major outcome of the *20dpfZF* assay was the indication of issues associated with the recovery of SLS sediment. Estrogenic responses in

fish species sampled from the SLS area, and exposed to similarly cored sediment, may confirm that assessment.

5.5.4 Comparison to alternative toxicity outcomes (III, IV)

In my studies I additionally used development in pre-hatch zebrafish embryos (1–3 dpf; III) and inhibition of bacterial luminescence reaction (III, IV) as endpoints to determine the gross toxic potential of elutriate from the contaminated sediments. Though these results are not comparable with the alteration of molecular biomarkers in the *20dpfZF*, owing to dissimilar MoAs, they highlight the significance of molecular signals for perception of toxicity in samples (III, IV).

The results from Study III showed that the survival of embryonic zebrafish was not affected by exposure to elutriate concentrations of HL and TBT-spiked sediments (> 90 % survival), being similar with the reference sediment (PJ). Instead, some ontogenic abnormalities were evoked by HL and TBT-spiked sediment elutriates relative to the water-control across developmental time (Fig. 15). Despite being a non-ecological setting compared to whole sediment assay with *20dpfZF*, the use of elutriate represent just another choice of experimental setting to assess potency of the sediment. However, the need of complementary studies for authentic ecotoxicological scenarios of ambient exposure is evident (Hyötyläinen and Oikari 1999). Importantly as well, the exposure settings were found to be sublethal as neither hatching rate nor survival of the embryos were different compared with the control groups (Fig. 15). Although Dong *et al.* (2006) showed developmental toxicity of TBT in zebrafish beyond 72 hpf, the hatching rate was not a sensitive endpoint for the TBT exposure in that study either. On the other hand, the same study (Dong *et al.* 2006) showed TBT-induced mortality from waterborne exposure at 30–50 µg l^{-1}, a concentration range which was apparently much higher than with TBT available from elutriate of either the HL or TBT-spiked sediments used in our study. Overall, however, it is commonly understood that the incidence of edemas and other malformations in developing zebrafish point to the embryo toxicity of contaminated sediments (Hollert *et al.* 2003, Wu *et al.* 2010). Altered blood circulation (heart rate) (Fig. 14) and ontogenic deformities, including delayed development and edemas of the pericardial and yolk sac regions (Fig. 15) were frequently seen in exposures of both HL and TBT-spiked sediment elutriates.

Sediment elutriates containing TBT and pure TBT showed inhibition of bioluminescence in *V. fischeri* (III). Overall, the bioluminescence toxicity of the *native* sample from Lake Hurulahti, HL-100, was probably in part due to TBT, but not exclusively. Additionally, e.g. MBT and DBT may have involved as well, being even more easily desorbed to the elutriate phase than TBT (Cheung *et al.* 2003). Furthermore, bioactive contents other than OTCs present in the authentic chemically contaminated sediment site of Lake Huruslahti could have contributed for the toxicity of HL-100 elutriate. Within the context of assessing

the toxicity of OTCs by zebrafish, given their targets at the cellular level (Pagliarani *et al.* 2013), more information were obtained from genomic responses in more developed life stages, i.e. *20dpfZF* exposed to OTC-contaminated sediment via water (III).

Elutriates of sediment sampled from Southern Lake Saimaa did not inhibit bacterial bioluminescence (IV), indicating non-toxic potential (unlike Lake Huruslahti, III). This finding accorded with earlier reports of SLS sediments collected from the same locations, by their being the least in surface layers (Lahdelma and Oikari 2005). While bioactive wood extractives were present in sediments (Lahdelma and Oikari 2005, 2006, Ratia *et al.* 2014), it seems possible that sensitive physiological pathways are responsive for modifiable gonadal and sex-differentiation in the *20dpfZF*. The whole sediment bioassays with polluted sediments of SLS revealed alterations in target gene expressions.

6 CONCLUSIONS

The aim of this work was (a) to identify and evaluate a practical, cost-effective and ontogenically-relevant model life-stage in zebrafish for assessing xenoestrogenicity associated with contamination of water and environmental samples, and (b) to identify relevant molecular biomarkers in the model and evaluate *in vivo* sensitivity to endocrine influence from environmental contamination. To accomplish that, molecular biomarkers mediated by ER in zebrafish were compared and selected. The work established water-borne toxicity to *in vivo* endocrine responses in zebrafish, but also included more complex exposure scenarios with sediment bioassays.

The primary studies (I) addressed the key objectives with the selection and use of the post-embryonic early juvenile zebrafish (*20dpfZF*) as a applicable life-stage using transcripts of *cyp19a1b* and *vtg1* as endpoints that were sensitive HPG-linked modulation, quantified at the molecular level using RT-PCR analyses. The *20dpfZF* life-stage provided for robust maintenance and experimentation, and responded to four model estrogens by corresponding induction patterns. The results of this study recommend the selection of *20dpfZF* as a representative life-stage including trophic transfer etc., for use in ongoing and further studies, including exposures to water-sediment interactions and mixtures of environmental chemicals.

Mixture-exposure studies (II) showed an additive *in vivo* effect on the transcription of key estrogenic genes in zebrafish (20 dpf) from a mixture of xenoestrogens. Among the studied genes, *vtg1* showed pronounced mixture-evoked transcript abundance compared with *cyp1a*, *cyp19a1b* and *esr1*. Further research is required for a broader understanding of the competitive mimics (xenoestrogens) with similar MOA. Furthermore, experimental designs should incorporate more realistic and complex mixtures with agonists, antagonists and dissimilarly acting chemicals (different MOAs).

In studies III and IV, the early-juvenile zebrafish (*20dpfZF*) was demonstrated as a potent life-stage expressing transcripts of *cyp19a1b* and *vtg1* as responsive endpoints to identify endocrine modulation by an environmental

site contaminated by industrial chemicals. The modulation of the key biomarkers, as observed with sediment with anti-estrogenic or androgenic (down-regulation) potential, presented a behaviourally intact *in vivo* model for screening industrially contaminated sediments. *In vivo* exposures also revealed a difference in transcript regulation between the authentic site loaded by OTCs and TBT, a dominant surrogate chemical. There was a down-regulation of *cyp19a1b* (aromatase), compared to the estrogen-sensitive up-regulation of *vtg1*. The assessment of contaminated surface sediment from Lake Huruslahti bay area also points to the complicated exposure scenarios with possible joint toxicity of co-contaminants. It is suggested that while studying contaminated sediment areas, relative comparisons should be carried out to several reference sites in order to ensure consistency of responses.

Pulp and paper industry-contaminated lake sediment inducted modulation of the brain aromatase gene *cyp19a1b* in the early juvenile zebrafish (*20dpfZF*) (IV). The estrogenic potential of weakly agonistic environmental samples was demonstrated by a change in transcript levels of *cyp19a1b*, but not of *cyp1a* (AhR-mediated) and *vtg1* (estrogen-responsive), despite the latter being also a potent estrogenic biomarker. The sensitivity of *cyp19a1b* regulation revealed alteration along the HPG axis, one of the key pathways leading on to endocrine disruption in exposed animals. Aromatase expression was identified as a sensitive endpoint, without vitellogenin regulation, acting on the sensitive stage of the sediment-exposed model, *20dpfZF*. The results also showed the up-regulation of *cyp19a1b* by the upper and recent sediment layers (0–5 cm) compared to two to three decades older ones (20–30 cm), indicating the required course of action which is often underestimated in the operations of paper mills for addressing endocrine toxicity.

As an overview, the use of *20dpfZF* addressed the requirement for a free-swimming, nutritionally independent early juvenile capable of assessing *in vivo* responses to endocrine-active substances, as shown in studies with artificial as well as naturally contaminated samples. The transcript abundance of key estrogen-responsive biomarkers is based on the altered gene transcription regulated by corresponding chemical exposure and post-exposure period.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Nuori seeprakala *20dpfZF* ekotoksikologisten uhkien mallieläimenä - väline hormonivaikutusten arviointiin

Kemikaalien ja päästöjen ympäristö- ja terveyshaitat halutaan ensisijaisesti välttää ennakolta. Terveysvaikutusten arvioimiseksi on viime vuosina voimakkaasti edistetty ns. vaihtoehtomenetelmiä koe-eläinten kipuun ja kärsimykseen liittyvien eettisten syiden takia. Uudet 3R-käytännöt pyrkivät vähentämään (reduce), korvaamaan (replace) ja parantamaan (refine) erityisesti selkärankaisten eläinten käyttöä ja vasteita, enenevässä määrin myös luonnonympäristöön kohdistuvien uhkien osalta. Luonnon systeemien monimutkaisuuden ja -muotoisuuden sekä tapauskohtaisuuden taustoja vasten on oletettavissa, että liian yksinkertaiset vaihtoehtomenetelmät aiheuttavat merkittävän epävarmuuslähteen ekotoksikologisten riskien arviointiin.

Työn tarkoituksena oli monipuolistaa ja luonnonmukaistaa yhä suositumman koe-eläimen seeprakalan (*Danio rerio*) käyttöaluetta mm. 3R-periaatteiden hengessä. Laboratoriokokein tutkittiin, miten hedelmöitymisestä lukien 20 päivän ikäinen varhaisnuori eläin eli *20dpfZF*-malli soveltuu teollisuuskemikaalien ja -sedimenttien hormonaalisten vaikutusten ennakointiin. Oletuksen mukaan tässä iässä seeprakalan toiminnallinen sukupuoli naaraana tai koiraana ei ole vielä lopullisesti määräytynyt. Osassa kokeita steroidihormonien säätelyyn lukeutuvien geenien ilmentymistä *20dpfZF*:ssä matalissa subletaaleissa pitoisuuksissa verrattiin kahteen muuhun toksisuuden 3R-tutkimustapaan, ts. bakteerin bioluminesenssin estoon ja seeprakalan varhaisiin alkiovaurioihin.

Tulokset osoittivat *20dpfZF*:n soveltuvan monipuolisesti estrogeenisten näytteiden arviointiin, mukaan lukien puhtaat esimerkkikemikaalit, niiden seokset sekä saastuneet teollisuussedimentit. Estradioliaineenvaihduntaa säätelevien kuuden geenin ryhmästä aromataasi *cyp19a1b* ja vitellogeniini *vtg1* osoittautuivat vasteiltaan suurimmiksi tunnetuilla ympäristöestrogeeneilla tehdyissä viiden päivän altistuksissa. Jatkotutkimukset kohdistettiin näihin kahteen geeniin, ja niiden vasteita mitattiin 3-5 vrk:n kestoisilla annos-vaste -kokeilla, jolloin yksittäisessä koeasetelmassa ja analyysinäytteessä oli 25 *20dpfZF*-yksilöä.

Seosvaikutuksen arvioimiseksi toisessa osatyössä *20dpfZF*-kaloja altistettiin usealle tunnetulle estrogeeniselle kemikaalille, joista kukin oli yksistään pitoisuudeltaan ei-vaikuttavaa tasoa. Seoksen kokonaisvaste geenitasolla oli kuitenkin selvä, erityisesti *vtg1*:n ilmentymisen aktivoiduttua. Johtopäätöksenä oli, että yhteisvaikutus on vähintään yksittäisten aineiden pitoisuuksien summa ympäröivässä vedessä, mistä seuraa, että seosten, kuten teollisuus- ja asutusjätevesien, estrogeenisuutta tulee arvioida kokonaisuutena. *20dpfZF* on käyttökelpoinen väline tähän tarkoitukseen.

20dpfZF:n geenivasteita kokeiltiin kahden erityyppisen teollisuussedimentin, OTC- ja PPM-sedimentin, estrogeenien aineenvaihduntaa muuntavan ominaisuuden tutkimiseen. Molemmissa saastuneissa sedimenttikohteissa on monilukuinen määrä mahdollisia hormonaalisesti vaikuttavia teollisuusperäisiä hait-

ta-aineita, OTC-sedimentissä erityisesti organotinayhdisteitä kuten tributyylitina (TBT) sekä PPM-sedimentissä puun uuteaineita kuten β-sitosteroli ja betulinoli. Tulokset osoittivat, että aromataasi *cyp19a1b*:n ja vitellogeniini *vtg1*:n vasteet sedimenttikokeissa erosivat toisistaan ja olivat luonteenomaisia alueelle, josta saastunut sedimentti oli peräisin. Molemmissa tapauksissa *20dpfZF* oli kuitenkin käyttökelpoinen väline sedimenttiperäisten hormonaalisten haittojen tunnistamiseen. Kehitetty koeasetelma lisäksi mukailee altistumisen luonnonmukaisuutta sedimentin vuorovaikutus-yhteydessä elävällä nuorella ja kasvavalla kalalla. Teollisuushistoriaa voidaan tarkastella myös ympäristöpolitiikan onnistumisen valossa tutkimalla ympäristönäytteitä sedimentin eri syvyyksiltä. Tutkitussa PPM-kohteessa, ts. kemiallisen metsäteollisuuden kuormittamalla alueella, alkuainekloorin käytön lopettamisen ekotoksikologiset hyödyt 1990-luvun alussa olivat myös *20dpfZF*-mallin valossa selvät. Toisaalta *20dpfZF*:n geenivasteet tunnistivat hormonaalisten vaikutusten uhkan kaloille aivan viime vuosinakin muodostuneen sedimentin vuoksi, huolimatta modernisoidusta sellu- ja paperintuotantoteknologiasta.

20dpfZF-malli on lupaava kustannustehokas väline yksittäisten kemikaalien, tuntemattomien kemikaaliseosten sekä monimutkaisten ympäristönäytteiden hormonaalisten vaikutusten arvioimiseen. Estrogeeniaineenvaihdunnalle oleelliset geenivasteet *20dpfZF*:ssä olivat yhtä herkkiä tai herkempiä kuin kaksi vaihtoehtoista 3R-tutkimustapaa. Geenivasteet ilmenivät vastaavissa subletaaleissa altistustasoissa, joita voi esiintyä teollisuuden tai asutuksen kuormittamissa luontokohteissa. *20dpfZF*-mallia tulisi kuitenkin kehittää eteenpäin, ja ensisijaisia jatkotutkimustarpeita on esitelty väitöskirjatyössäni.

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ORIGINAL PUBLICATIONS

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Use of early juvenile zebrafish *Danio rerio* for *in vivo* assessment of endocrine modulation by xenoestrogens.

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Research Article Open Access

Use of Early Juvenile Zebrafish *Danio rerio* for *In-Vivo* Assessment of Endocrine Modulation by Xenoestrogens

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Abstract

Reliable and cost-effective early-life stage (ELS) bioassays incorporating practical experimentation without compromising scientific relevance are crucial in chemical risk assessment. This study investigated the use of 20 days-post-fertilization life stage (*20dpfZF*) of zebrafish *Danio rerio* to screen environmental chemicals known to be estrogenic in adult fish. Firstly, studies with key genes in steroidogenesis were conducted; the brain isoform of aromatase gene (*cyp19a1b*) being the most prominently expressed biomarker. Regulation of mRNA levels of molecular biomarkers, vitellogenin 1 gene (*vtg1*) and *cyp19a1b* were selected to assess the endocrine modulation by xenoestrogens, 17α-ethinylestradiol (EE2), 4-*n*-nonylphenol (NP), 4-*t*-octylphenol (OP) and bisphenol A (BPA). Groups of *20dpfZF* (n=15) as three replicates were exposed to chemicals over a five-day period in aerated static setups. Exposure of *20dpfZF* to sediment spiked with EE2 (nominal 3µg g^{-1}dw) was also conducted to assess the sensitivity of this life-stage to sediment with estrogenic potency. Whole body homogenates of exposed juveniles showed the estrogenic potential of chemicals in the order: EE2 > OP > BPA > NP. Higher relative expression of *cyp19a1b* was noticed at lower ambient concentrations of EE2, although *vtg1* showed more pronounced expression to it. The *20dpfZF* responded in a dose-related way to sediment spiked with EE2, expanding its use as a general aquatic animal model. The suitability of *20dpfZF* as an *in vivo* model, along with stable expression of reference genes was established. In addition to consistent expression pattern of key target genes on xenoestrogenicity, it serves as a practical screening model for the risk assessment of environmental chemicals and samples with estrogenic potential.

Keywords: Brain aromatase; Early-life stage; Vitellogenin1; Xenoestrogenicity; Zebrafish

Introduction

Diverse groups of natural and synthetic compounds known to have an endocrine bioactive potential are constantly introduced into the environment. These endocrine disrupting compounds (EDCs) have been found to exhibit significant effects on reproduction by acting on endocrine functions [1,2]. Given the extent of organic pollutants released into aquatic environment, teleosts among vertebrate taxa qualify as candidates to investigate endocrine modulation [2-6]. The choice of zebrafish *Danio rerio* (Hamilton, 1822) as a toxicological model presents practical advantages, including cost-effective maintenance, asynchronous breeding, and rapid development of the embryo-larval stages. Moreover, the sexual differentiation in the brain determining the fate of the gonads in teleosts, contrasts with amphibians and mammals [7]. In general, signaling cues in the form of endogenous hormones and external factors stimulate the pituitary to release gonadotropins, which in turn stimulate the gonads to synthesize and release endogenous hormones, sex steroids. Under endocrine control, the hypothalamus-pitutary-gonadal (HPG) axis is further extended to the liver (HPGL), the site for synthesis of the precursor of yolk protein, vitellogenin (*Vtg*), common to oviparous vertebrates. The hepatic synthesis of these proteins is stimulated by the binding of physiological estrogens or xenoestrogens to estrogen receptors (ERs) in the liver (Figure 1). In zebrafish, seven isoforms of *vtg* have been identified, with dominant expression of *vtg1* compared to *vtg*2-7 [8]. Males possessing the normally unexpressed *vtg* genes when exposed to exogenous estrogen or their mimics, up-regulate vitellogenesis [1]. Consequently, alteration of endocrine signaling along the HPGL axis and subsequent modulation of gametogenesis and other reproductive processes from teleost responses indicates mechanisms of EDC action [9].

Bio-chemical pathways have been used to link health and reproductive status in wildlife to environmental chemical exposures. Steroid biosynthetic enzymes catalyze the conversion of cholesterol into active sex hormones (estradiol and testosterone) mainly in brain, kidney and gonads [10]. Key enzymes in steroidogenesis include steroidogenic acute regulatory (Star) protein, cytochrome P450 side-chain cleavage (P450scc) or Cyp11a1, 3-beta-hydroxysteroid dehydrogenase (Hsd3b1), and cytochrome P450 aromatase (P450arom) or Cyp19a1 (Figure 1). Other studies have also discussed regulation in the steroidogenic pathway with gene expression analyses with adult fathead minnow [11] and zebrafish (*in vitro*, [12]; *in vivo*, [13,14]. Immunohistochemical studies showed the significance of (endogenous) sex steroids on gonadal sex differentiation in teleosts [9,15]. Other studies did not support the involvement of endogenous steroids in the gonadal sex differentiation in fish species such as medaka [16]. Therefore, it was interesting to compare in this study the expression patterns of key steroidogenic enzymes in early juvenile zebrafish, if any. Importantly, the synthesis and release of sex steroids is controlled by the aromatization of C19 androgens (testosterone) to C18 estrogens (17β-estradiol) by aromatase. In teleosts, the expression of tissue-specific isoforms of P450arom genes (gonad, *cyp19a1a;* brain, *cyp19a1b*) [17], with significantly higher relative expression of the brain isoform, has been used to indicate estrogenic stimulation of sex steroid synthesis [18-23]. Besides induction of *vtg1*, alterations of steroid biosynthesis and plasma steroids are biomarkers indicative of estrogenic action [24].

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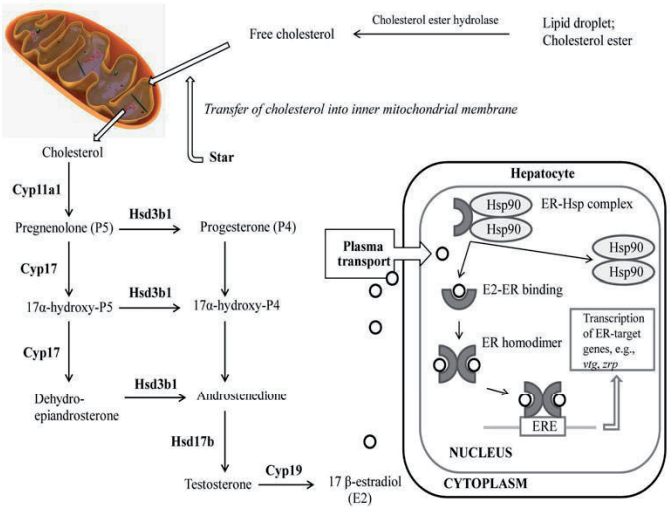

Figure 1: An outline of the steroid biosynthetic pathway and the action of estrogen on vitellogenesis in fish and other vertebrates**.** The conversion of cholesterol to pregnenolone and subsequent steps occurs in the inner mitochondrial membrane. Homodimerization of ERs and binding to estrogen-responsive elements (ERE) in the regulatory region (promoter) of susceptible genes lead to transcription of estrogen-responsive genes.

Along with *vtg1*, gene expression of *cyp19a1b*, a central steroidogenic enzyme, present suitable molecular markers to detect and analyze the impact of estrogenic exposure, considering the short ovarian cycles and estrogen-responsive embryonic stages in zebrafish. The post-embryonic life-stage (20 days-post-fertilization zebrafish; *20dpfZF*) was used for a five-day exposure to 17α-ethinylestradiol (EE2), a common benchmark of xenoestrogens. Although ranging in nano-and micro-molar concentrations in ambient water (0.2-40.0 ng L^{-1}; [25]) and sediment (< 0.5-22.8 μg kg^{-1} dry mass; [26]) sources respectively, EE2 is a potent xenoestrogen known to have adverse effects on the endocrine function of aquatic organisms.

The objective of this study was to evaluate a life-stage incorporating practical and experimental advantages for screening environmental estrogens, assessed by gene expression analyses. Pilot exposures to EE2 were carried out to select the key steroidogenic enzyme, *cyp19a1b* to complement *vtg1* as a panel of estrogen-responsive biomarkers for subsequent studies. Besides EE2, the sensitivity of this life-stage was evaluated with water-borne exposures of other xenoestrogens with varying potencies-bisphenol A (BPA), 4–*n*–nonylphenol (NP) and 4-*tert*-octylphenol (OP). The screening potential of *20dpfZF* was also verified with exposure to sediment spiked with EE2, and is discussed in context to regulatory requirements with the use of adult animals in toxicological research.

Materials and Methods

Chemicals and exposure stock solution

2,2-bis(4-hydroxyphenyl)propane (BPA, purity 99+%), 17α-ethynyl,3,5(10)-estratriene-3, 17β-diol (EE2, minimum 98% by HPLC), and 4-(1,1,3,3-tetramethylbutyl)phenol (OP, 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-n-Nonylphenol (NP, 98+ %) was obtained from Alfa Aesar (Karlsruhe, Germany). Silylation reagent N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from Fluka Chemie (Buchs, Switzerland). Internal standards 17-α-ethynylestradiol-d4 (EE2-d4, 97-98%) and 2,2-bis(4-hydroxyphenyl) propane-d16 (BPA-d16, 98%) were purchased from LGC Standards AB (Borås, Sweden). The solvents, methanol (MeOH) and acetonitrile, supplied by Merck Chemicals (Darmstadt, Germany) were of HPLC grade. Separate chemicals were dissolved in methanol, and stocks were stored at -20°C until use, within two weeks.

Experimental animals

Adult male and female zebrafish (*Danio rerio*) (wild type; WT) were obtained from Institute of Medical Technology, University of Tampere (Tampere, Finland). Fish were acclimatized to temperatures (mean ± standard deviation, 26 ± 1°C) and photoperiod (14 h light/10 h dark) for at least two weeks prior to setup for breeding. Animal husbandry was carried out along established guidelines (Westerfield, 2007). Zebrafish adults were fed twice daily with frozen chironomid larvae (Ruto frozen fish food, Zevenhuizen, Holland). Following breeding between sexually mature adults (~8 month old), fertilized eggs (0.5-1.5 hours-post-fertilization; hpf) were collected and reared through early post-embryonic stages for 20 days (*20dpfZF*) in rearing water or embryo medium (Westerfield, 2007). Post-hatch zebrafish from 6 dpf through 25 dpf were fed with fry food (SDS diets, UK).

Chemical exposure and sampling

Chemical exposures were conducted in glass jars containing 250 mL of exposure medium, consisted of gently aerated embryo-rearing water (pH 7.2) for the duration of the experiment, to avoid change in water quality. Water-borne exposures (I and II) were carried out in a semi-static manner, replacing 100% of the medium daily. Water quality parameters were measured on days 1 and 4. The mean temperature in the exposure jars was in the range 24-26°C. The content of dissolved oxygen (DO, mg L^{-1}) was 7.6 ± 0.2 (mean DO ± sd) and pH 7.2 ± 0.2 (mean ± sd). Chemicals dissolved in the carrier solvent, methanol (MeOH), were administered to the exposure water, the solvent concentration being 0.02% or less. Preliminary exposures also included water controls (data not shown), i.e., without solvent to confirm similarity with that of the maximum solvent concentration (0.02%). The first set of exposures (Exposure I) were performed with groups of 30 fish (*20dpfZF*), replicated three times in parallel, exposed to 0.02% MeOH with 0, 5, 25 and 50 ng EE2 L^{-1}, to determine expression and selection of suitable of steroidogenic gene(s). The second set of exposures (Exposure II) were performed with groups of 25 fish (*20dpfZF*), replicated three times, exposed to 0.02% MeOH with 0, 5, 25, and 50 ng EE2 L^{-1}, 0, 100, 500, and 1000 µg BPA L^{-1}, 0, 10, 50, and 100 µg NP L^{-1}, and 0, 10, 50, and 100 µg OP L^{-1}. Although the results used in discussion represent measurement from three parallel replicates, the pattern of relative expression of target genes were also verified with two independent exposures conducted earlier. Fish were sampled at the end of five days of exposure. Each group of sampled animals (n = 15) was collectively weighed in rnase-free micro centrifuge tubes (Starlab, Germany), and snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Experiments were conducted according to and licensed by the Finnish authority for animal experiments (ESAVI-2010-07885/Ym-23).

Sediment exposure

The reference sediment (0-10 cm depth; dry weight, dw 5.2%; total organic content, TOC 4.7%) was sampled from Lake Palosjärvi (located in Central Finland) using an Eckman grab sampler. Chemical spiking of it was performed according to standard guidelines for whole-sediment toxicity testing [27,28]. Briefly, nominal concentration 3 µg g^{-1} dw of EE2-spiked sediment was prepared by gradually administering EE2 (dissolved in 100% methanol) to sediment slurry with minimum amount of water mixed for ~7 h. Following the addition of EE2, the headspace of sample container was filled with an inert gas (N_2) and sealed for equilibration at 4°C for a minimum of 30 d before the assays. The exposure series (Exposure III) were prepared in 600 mL glass beakers in a ratio 1:4 (dw/v) of sediment and overlying water (3°dH or 54 mg $CaCO_3$ L^{-1}), added slowly to avoid any suspension of particulates to the water column. The exposure beakers were allowed to stand for ~16 h at 24-26°C before the experimental fish (n=25) were added. The exposure series consisted of control sediment (Ref-Sed; i.e., the reference Palosjärvi sample) compared with EE2-spiked samples; Sed 1 (3 d), and Sed 2 (5 d). The exposures of animals were conducted in aerated static systems (no water renewal), sampled at the end of 3 d for Ref-Sed, Water-EE2 (150 ng L^{-1}), Sed 1+ Salt and Sed 1, and at the end of 5 d for Sed 2 (Figure 4). Two controls treatments, each accompanying the respective 3- and 5 d exposures were included to provide for relative quantification per sampling time point. To maintain the water quality, the fish were not fed during the experiment. To assess the possible role of the influence of electrolyte content of overlying water, another sediment exposure Sed1 + Salt included E3 salts (embryo medium; 6°dH or 107 mg $CaCO_3$ L^{-1}) at levels used for rearing of embryo-larval stages of zebrafishup to *20dpfZF*. For comparison of estrogenic effects, water-borne exposure of *20dpfZF* to 150 ng EE2 L^{-1} for 3d was also included as positive control. Experimental conditions and fish sampling (n=3 pools of at least 15 fish each) was the same as in water-borne exposures.

Solid-phase extraction (SPE) of water samples

Ethinylestradiol was extracted from water samples by a SPE method [29] modified for the analyses in the present study (see supplementary information). Similar extraction was employed with NP and OP samples (3-10 mL) using BPA-d16 as the internal standard.

GC-MS analysis of EE2

The gas chromatography–mass spectrometry (GC-MS) was performed with HP 6890 gas chromatograph (Hewlett-Packard, Walbronn, Germany) equipped with HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, CA, USA). The method included a derivatization [30] by silylation with BSTFA, and pyridine to increase the stability of derivatized products and improve extraction recoveries of the target compounds (see supplementary information). Concentration of EE2 was calculated using relative spectral peak-areas of analytes, internal standard, and the determined response factor. Specifically, the results were based on determination of di-TMS derivatives of EE2 and EE2-d4.

LC-MS /MS analysis of BPA, NP, and OP

The analysis was performed with Waters Alliance 2795 (MA, USA) LC and the determination of target compounds was performed in negative ion electro spray mode (ESI-) and a Quattro Micro triple-quadrupole mass spectrometer (MS/MS) (Waters, MA, USA) with electro spray interface was used as detector. The method described by Revilla-Ruiz et al. [31], with minor modifications, was conducted with extracted (NP and OP) and un-extracted (BPA) water samples. Data acquisition was performed with multiple reactions monitoring (MRM) mode and the corresponding parameters for the target compounds are presented in supplementary (Table SI).

Isolation of RNA and reverse transcription

Total RNA was extracted from homogenized pooled fish samples (15 animals with total weight ca. 16 mg) using the Tri Reagent (Molecular Research Center, Inc.) according to manufacturer's instructions, and the quality and quantity were determined on a NanoDrop ND-1000 UV-visible spectrophotometer. Absorbance measurements including 260:280 ratios (1.8 to 2.0) and the additional 260:230 > 260:280 ratios indicated a secondary measure of nucleic acid purity [32]. The integrity of RNA was also verified using the Agilent RNA 6000 Nano Assay Protocol with an Agilent 2100 Bioanalyzer (Waldbronn, Germany). RNA integrity number (RIN, 1-10) generated for analyzed samples was checked within range 7-10 for all samples. The total RNA extracted was reverse transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad, USA), and the cDNA templates were diluted ten times for use in real-time PCR assays.

Real-time PCR

Real-time PCR was performed using iQ SYBR Green supermix (Bio-Rad, USA) and run on a Bio-Rad CFX96™ real-time PCR detection system (Bio-Rad, USA). Gene-specific primers (Table SII) for house-keeping genes (beta-actin (*β-actin*) and elongation factor 1 alpha (*ef1a*)) as well as target genes (*star, cyp11a1, hsd3b1, cyp19a1b*) were designed using Amplifx (v 1.5.4), and the given oligonucleotides (desalt purified) were purchased from Sigma-Aldrich (UK). The primer pair sequences were checked with the program Mfold (http://mfold.bioinfo.rpi.edu/

cgi-bin/dna-form1.cgi) to predict the potential for amplicon secondary structures that may prevent efficient amplification. To evaluate qPCR efficiency, amplification efficiency with standard curve analysis (PCR efficiency, 90-110%; slope, -3.1 to -3.6; $R^2 > 0.98$) was performed, and optimization of qPCR reactions and protocol was carried out. The qPCR reaction mix was optimized for a 25 µL reaction mix consisting of 12.5 µL of SYBR green supermix, 2.5 µL of each of the primer pairs (200 nM), 2.5 µL sterile water, and 5 µL of cDNA template (100 ng μL^{-1}). The PCR protocol comprised 10 min polymerase activation at 95°C, followed by 40 cycles of 30 sec at 95°C, and 30 sec at 60°C. Each sample was assayed in duplicate, and assays requiring multiple runs included inter-run calibrators (IRCs) on each of the plates to facilitate sample maximization and restricting reference gene reactions to a single plate. The inter-run calibration was performed to correct for variation, allowing comparison between plates within the same analysis.

Data analysis

The expressions of target genes were normalized to the expression of two reference genes, (*β-actin*, *ef1a*). The choice of reference genes was based on their stable expression across samples of different treatment. The determined stability parameter (M value) for the reference genes were used to select suitable reference genes with M values < 0.5; the lower the M-value, that higher the stability [33]. The relative gene expression levels were calculated from the experimental amplification data (Cq values; quantification cycles) using the $2^{-\Delta\Delta CT}$ method [34]. Statistical significance (P< 0.05) of log-transformed data from Exposures I and III was determined by a one-way analysis of variance (ANOVA) followed by Tukey HSD *post hoc* test. Data from Exposure II was analyzed using Welch's test followed by Games-Howell *post hoc* test. All analyses were performed using IBM SPSS (v 19).

Results

Water quality and concentrations of xenoestrogens in experiments

No significant difference in water quality was found between exposure jars or treatments during the exposure period. Regarding to nominal concentration added in experimental waters, actual chemical concentrations (mean ± sd) are presented in Table SIII. However, all measured concentrations of NP were not detected. Since both NP and OP samples were extracted simultaneously with the same method, the undetected NP samples may have resulted from issues with the extraction method. For clarity, while the measured concentrations commonly ranged 20-50% below expected, revealing effects, the nominal values are used in further discussion.

Exposure I: Responses of steroidogenic enzyme mRNA levels

The mean M-values of reference genes, *β-actin* and *ef1a* across the corresponding concentration series of exposures (BPA, NP, OP, and EE2) were 0.004, 0.1, 0.3, 0.06; and 0.01, 0.04, 0.06, 0.09 respectively, showing stable expression of the respective housekeeping genes. For normalization of gene expression, the mean M-values of both reference genes combined and considered together for the chemical exposures were 0.01, 0.07, 0.06, and 0.07 respectively, suggesting the stable expression of this reference gene pair across chemical treatment at the studied life-stage.

Figure 2 shows the normalized gene expression levels of *cyp11a1, cyp19a1b, hsd3b1,* and *star* in *20dpfZF* exposed to EE2 for five days (20-25 dpf). Among the studied genes, only *cyp19a1b* showed pronounced expression levels compared with *cyp11a1, hsd3b1,* and *star,* (Tukey HSD, P< 0.001). Noticeable induction of *cyp19a1b* resulted formal EE2 exposures (5, 25 and 50 ng L^{-1}), averaging 13.5, 18.7 and 22.8-fold changes respectively, with gradual increase with increasing concentrations (5-50 ng L^{-1}). In preliminary studies with similar experimental conditions, *cyp19a1b* showed ~2-fold increase at even lower exposures (1 ng L^{-1}) (data not shown). There was no induction of *star* in exposure groups, with expression even in the highest EE2 exposure, 50 ng EE2 L^{-1}, being similar with that of control and 5 ng EE2 L^{-1}. Although 25 ng EE2 L^{-1} revealed ~1.3-fold change, the altered *star* expression was not significantly different from either the control or respective expression levels of *cyp11a1* and *hsd3b1* (Tukey HSD, P > 0.05). Similarly, no statistically significant effects were observed with the mRNA levels of *cyp11a1* and *hsd3b1* that actually showed decreased average expression compared to solvent control. Overall, there was a statistically significant difference between expression of *cyp19a1b* and the other steroidogenic enzymes (*star, cyp11a1* and *hsd3b1*) as determined by one-way ANOVA for EE2 exposures 5 ng L^{-1} ($F(3, 8) = 91.740$, P< 0.001), 25 ng L^{-1} ($F(3, 8) = 61.130$, P< 0.001) and 50 ng L^{-1} ($F(3, 8) = 133.082$, P< 0.001). A Tukey HSD *post hoc* test revealed the significant up-regulation of only *cyp19a1b* expression among the studied steroidogenic enzymes (P< 0.001) compared to *star–cyp11a1* (P = 0.848) and *star–hsd3b1* (P = 0.982).

Exposure II: Changes in *cyp19a1b* and *vtg1* mRNA levels to xenoestrogens in water

Figure 3a shows the relative expression of *cyp19a1b* and *vtg1* in *20dpfZF* exposed to EE2 from 20 through 25 dpf. The mRNA levels of *cyp19a1b* exposed to 5, 25, and 50 ng EE2 L^{-1} were induced to 13, 19, and 24-fold respectively, relative to control treatment. For the same concentrations, relative expression levels of *vtg1* were 5, 143, and 1272-fold. Despite the larger extent of *vtg1* expression across the exposure treatment, *cyp19a1b* showed more sensitive induction (13-fold; Games-Howell, P< 0.05) at the lowest range (5ng EE2 L^{-1}) compared to *vtg1* (5-fold; Games-Howell, P< 0.05). As presumed, the pattern of expression of *cyp19a1b* at all concentrations of EE2 was similar to samples studied for steroidogenic enzyme mRNA levels (Figure 2d); with gradual increase from 5 to 50 ng EE2 L^{-1}. However, at higher treatments levels (25 and 50 ng EE2 L^{-1}), the relative induction of *vtg1* (Games-Howell, P< 0.01, 0.001) was noticeably higher than *cyp19a1b* (Games-Howell, P< 0.05), albeit both the genes showing significant relative expression. Almost 15% mortality was observed in groups exposed to the highest nominal concentration of EE2 (Figure S1).

Whole-body *cyp19a1b* showed increased expression levels at 100, 500 and 1000 µg BPA L^{-1} compared to *vtg1* (Figure 3b). Expression of *vtg1* was down-regulated (in average -1.2-fold change) by lower concentrations of BPA (100 and 500 µg L^{-1}), but was significantly up-regulated at the highest concentration (2.7-fold change; Games-Howell, P< 0.05). The two higher concentrations of BPA L^{-1} showed significant induction of *cyp19a1b* transcripts, 2.9 and 4.4-fold (Games-Howell, P< 0.05) respectively. Clearly, *cyp19a1b* was more sensitive compared to *vtg1* across a range of BPA exposures, further revealing that BPA induces a weak estrogenic effect when compared to EE2.

In *20dpfZF* exposed to 10, 50 and 100 µg NP L^{-1}, the relative expression levels of both *cyp19a1b* and *vtg1* showed no statistically significant change (Games-Howell, P> 0.05), showing a clear down-regulation for both genes (Figure 3b). Compared to NP, the expression pattern was different with OP exposure; *cyp19a1b* showing an up-regulatory pattern for all three concentrations compared to control (Figure 3b). The relative induction of *vtg1* was significant only at 100

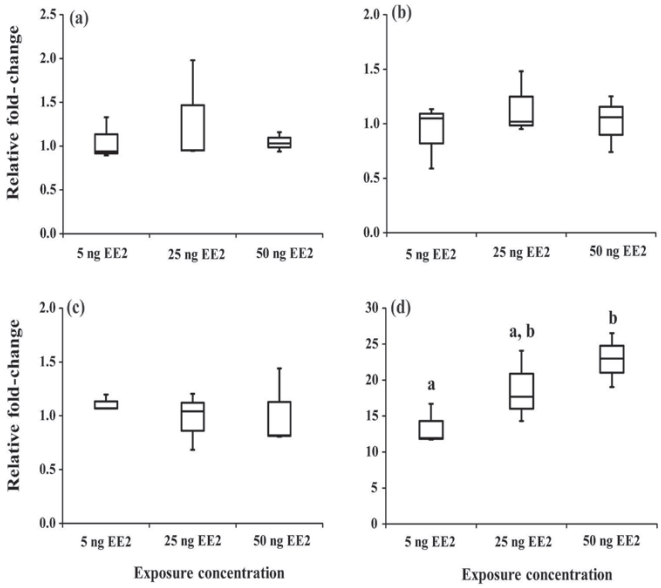

Figure 2: Exposure I: Changes in mRNA levels of steroidogenic enzymes. Gene expression of *star* (a), *cyp11a1* (b), *hsd3b1* (c) and *cyp19a1b* (d) in *20dpfZF* exposed to concentrations of EE2 for five days (from 20 to 25 dpf). Box plots represent median (—), 25th and 75th percentiles (lower and upper end of boxes), minimum and maximum values (end of the whiskers) for polled whole-body homogenates (n=3 pools of at least 20 fish each). Target genes were normalized to geometric mean of two reference genes (*β-actin*, *ef1a*). Nominal exposure concentrations include 0, 5, 25 and 50 ng EE2 L^{-1}. Means with different letters are significantly different ($P< 0.05$) as determined by One-way ANOVA followed by Tukey's HSD *post hoc* test.

µg OP L^{-1} (2.5-fold; Games-Howell, $P< 0.05$). For all the concentrations, *cyp19a1b* again showed higher sensitivity for the induction, i.e., 1.9, 1.7, and 2.5-fold. With OP, as in the case of EE2 and BPA, *cyp19a1b* was a more sensitive biomarker of estrogenicity than *vtg1*. Overall, it is noteworthy that NP compared to EE2, BPA, and OP had a much less estrogenic effect in the *20dpfZF* model. There was statistically significant difference between exposure groups determined by Welch's test for both *cyp19a1b* (F (11, 9.197) = 422.008, $P< 0.001$) and *vtg1* (F (11, 9.355) = 666.860, $P< 0.001$). Both 50 and 100 µg OP L^{-1} affected the survival of exposed fish to ~20 and 30% mortality (Figure S1).

Exposure III: Changes in *cyp19a1b* and *vtg1* mRNA levels to sediment spiked with EE2

In relevant contexts of *in vivo* exposures, *20dpfZF* showed preferential use of water-column depending on exposure type (with and without sediment). While each group of 25 animals in the water-only units appeared to distribute randomly across the water column, the fish in units with sediment headed for the sediment-water interface, perhaps seeking food. Actually, by actively ingesting contaminated sediment, the possibility to study diverse exposure scenarios with *20dpfZF* (i.e. contaminants taken up by ingestion) was obvious (Figure 5b). We suggest that the same is not possible with sessile and non-feeding life stages.

One-way ANOVA analysis of normalized gene expression showed significant induction of both *cyp19a1b* (F (4, 10) = 1096.501, $P< 0.001$) and *vtg1* (F (4, 10) = 2471.846, $P< 0.001$) of *20dpfZF* when exposed to sediment spiked with nominal 3 µg EE2 g^{-1}dw over three to five days (Figure 4a, 4b). Between the exposures (Sed1 and Sed2) conducted over three and five days, Tukey's HSD *post hoc* test showed that fish exposed to sediments with EE2 exhibited higher induction of *cyp19a1b* than the water-borne exposure (nominal 150 ng EE2 L^{-1}) at 63- and 50-fold change, respectively (Figure 4a). Significant up-regulation of Sed 2 fish with 74-fold change, compared to EE2-water exposure, indicated the influence of the extended exposure duration. For the 3d exposures (Sed1+Salt, Sed1), which differed only in respect to the electrolyte content, showed higher induction (72-fold) compared to that of Sed1, although not significantly different (Tukey's HSD, $P = 0.473$).

The expression of *vtg1* showed very pronounced up-regulation patterns, increasing from EE2 in water (181-fold) to Sed 1 (500-fold) and Sed 2 (1152-fold) (Figure 4b). Unlike *cyp19a1b*, *vtg1* showed significantly lower induction in Sed 1 + Salt (198-fold) when compared to Sed 1 (500-fold), possibly as an indication of the difference in the fraction of EE2 taken up by the fish (Tukey's HSD, $P< 0.001$). These may be considered consistent with differences in the partitioning and bioavailability of EE2 between static water and sediment exposures systems, based on ambient characteristics.

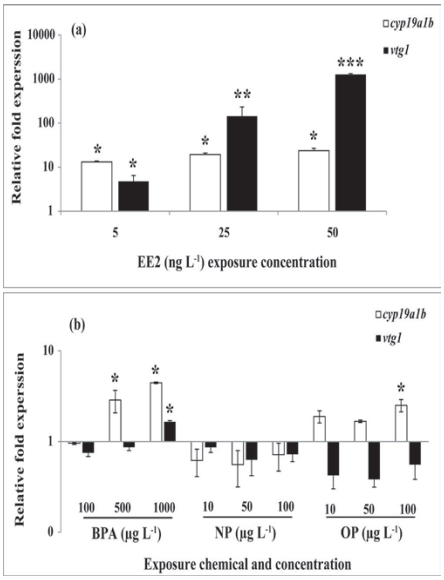

Figure 3: Exposure II: Xenoestrogen-induced gene expression in *20dpfZF*. Fold-change expression ofcyp19a1b andvtg1 in *20dpfZF* exposed to three nominal concentrations of EE2 (a), BPA, NP and OP (b) for five days, i.e., over the period 20–25 dpf, in a whole-body assay. Bars represent gene expression fold-change (± SD; n = 3 pools of 15 fish each) normalized to geometric mean ofβ-actinand ef1a and relative to solvent (0.01% methanol) control. The expression levels are in log10 scale. Note expression of both cyp19a1b and vtg1 genes were down-regulated in NP exposure (b). The asterisks represent statistically significant differences, * P< 0.05, ** P< 0.01 and ***P< 0.001 as determined by Welch's test followed by Games-Howell post hoc test.

Discussion

Among aquatic vertebrates, the zebrafish has been widely applied in investigating biological responses in development [35], endocrine disruption [3,4,36], and reproduction [37] among other effects. As adult organisms, the fish has been established as a reliable and sensitive organism in studies of comparative toxicology. On the other hand, in line with alternative animal testing requirements, ELS bioassays are aimed to substitute more advanced stages for toxicity testing [37-39]. The feasibility to expose ELS includes a large sample size, not possible with adult fish. Moreover, an early *in vivo* model presents an integrated organism system compliant with regulatory mandates of vertebrate animal testing. The reliability of gene level responses [40,41], including estrogenic and the other steroidogenic ones, is suggested to give a mechanistic understanding on impacts of environmental chemicals, their dynamics of effects, and most importantly, extrapolations used in environmental risk assessment. Introduction of the *20dpfZF* clearly satisfied several scientific, practical, and cost-effective requirements, although the main focus of this study was to select and evaluate the life-stage as a suitable screening model for xenoestrogenicity. The information regarding the gene-level response determined here in water-based exposures could be compared to a sediment exposure carrying a potent xenoestrogen with substantial trait of hydrophobicity, EE2 (log K_{ow} 4.15) [42].

Exposure of zebrafish (20-25 dpf) to EE2, BPA, NP, and OP was used to evaluate regulation of *cyp19a1b* and *vtg1* transcript levels. The choice of *cyp19a1b* as a core endpoint for this life-stage was determined from concentration-specific transcript abundance compared to other steroidogenic enzymes (*star, cyp11a1* and *hsd3b1*; Figure 1). The presence of ERE in the promoter region of *cyp19a1b*, but absent on *cyp19a1a*, has been shown to explain the estrogen-responsiveness of *cyp19a1b* [7,18,43].

Exposure I: Selection of steroidogenic endpoint for xenoestrogenicity

The steroid biosynthetic pathway can be a susceptible target because of its key enzymes, each capable of setting off a cascade of events. For instance, in adult zebrafish exposed to water-soluble fraction of crude oil, Arukwe et al. [13] demonstrated a negative relationship between altered steroidogenic responses and xenobiotic biotransformation processes. The same study showed down-regulation of important steroidogenic enzymes including *star* and *cyp11a1*, and up-regulation of *hsd3b1* along with increasing contaminant level. Levi et al. [14] showed hepatic expression of *star* in non-vitellogenic female zebrafish. In the present study we found no change of *star* mRNA levels except some tendency for down-regulation relative to the control group at exposure levels 5 and 25 ng EE2 L^{-1} (Figure 2a). Filby et al. [11] also showed down-regulation of *star* mRNA in adult male fathead minnow exposed to 10 ng EE2 L^{-1} but no significant changes in exposed females. *In vitro* cultures of zebrafish ovarian follicles at different maturation stages exposed to human chorionic gonadotropin revealed an increase in expression of *star*, *cyp11a1* and *hsd3b1* with mature stages, in contrast with a decreasing pattern without exposure [12]. In this study, the mRNA levels of *cyp11a1* and *hsd3b1* were relatively lower for all exposure concentrations with lowest relative expression for 25 ng EE2 L^{-1} at 59% and 45% respectively (Figure 2b). Lower expression levels of *star*, *cyp11a1*, and *hsd3b1* in fish exposed to EE2, a potent agonist for estrogenic effects, suggest effective regulatory processes in effect. In comparison to three other transcripts, *cyp19a1b* showed a clear dose-specific up-regulation pattern. Filby et al. [11] that showed up-regulation of only *cyp19a1a* but not *cyp19a1b* in male fathead minnow exposed to EE2, possibly owing to the pronounced expression of the brain isoform (*cyp19a1b*) in earlier life-stages. The use of *cyp19a1b* signal has also been employed in transgenic zebrafish specifically as estrogenic-sensitive screens [44,45]. Because *cyp19a1b* is a key player in steroid biosynthesis at stage of *20dpfZF*, involved in conversion of androgen to estrogen, it qualified as a compelling signature of xenoestrogen-induced effects.

Exposure II: *cyp19a1b* and *vtg1* mRNA regulation by four potential xenoestrogens

In the present study, modulation of *cyp19a1b* and *vtg1* mRNA expression was used as biomarker for estrogenic effect in the identified responsive ELS of zebrafish (Figure 3). Earlier, in mature adults, estrogen-mediated expression of *cyp19a1b* has been described among other teleosts, e.g. in Atlantic salmon [46], mangrove killifish [47], medaka [48], and zebrafish [18-20]. Expression of *cyp19a1b* in developing zebrafish (3-4dpf) was shown to be a sensitive indicator of estrogen-induced effects even earlier than *20dpfZF* [49]. Giving further support to our research, Muncke and Eggen [50] described the induction of *vtg1* mRNA as a reliable endpoint for 1-5 dpf zebrafish, presenting alternatives for adult animal testing.

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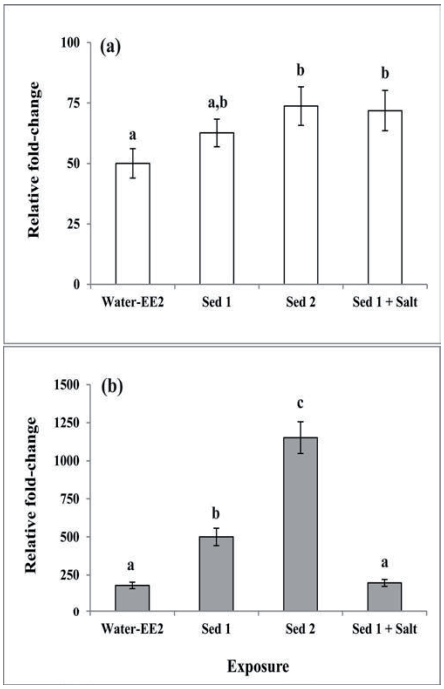

Figure 4: Exposure III: Gene induction caused by sediment-borne EE2. Foldchange of *cyp19a1b* (a) and *vtg1* (b) from whole body homogenate of early juvenile zebrafish (23–25 dpf) exposed to sediment spiked with EE2. Fish (n = 3 pools) were sampled at the end of 3 d for Ref-Sed, Water-EE2 (150 ng L-1), Sed 1+Salt and Sed 1 and at the end of 5 d for Sed 2. Bars represent normalized gene expression expressed as fold-change relative to control or reference exposure (Ref-Sed). Values are mean ± SD (15 fish per triplicate pool). Means with different letters are significantly different (P< 0.05) as determined by One-way ANOVA followed by Tukey's HSD *post hoc* test.

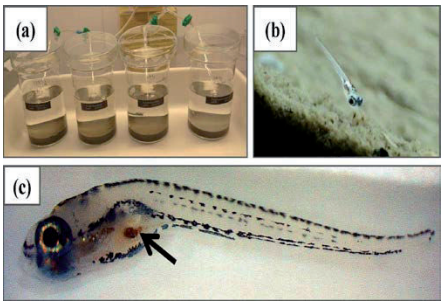

Figure 5: Experimental setup of sediment assay (Exposure III) over 5 day exposure period (a). Free swimming juvenile zebrafish (20-25 dpf) foraging on particulate matter in upper most sediment layer (b). Arrow indicates ingested sediment in accumulated in the gut of fish (body length, 7.8 mm) at the end of exposure (c).

Our juvenile zebrafish (20 dpf) exposed to EE2 showed inductions of *cyp19a1b* and *vtg1* in a concentration-dependant manner, *vtg1* being more distinct than *cyp19a1b*. Importantly, however, the expression levels of *cyp19a1b* were increased in all exposure groups relative to control, serving as an excellent benchmark. For comparison, in adult male zebrafish exposed to EE2 for three weeks, induction of *cyp19a1b* mRNA [51] and protein levels [4] were also evident. Our data with *20dpfZF* showed around 13-fold *cyp19a1b* induction at nominal concentration of 5 ng EE2 L^{-1} and more at higher concentrations, keeping with range of expression in adults. Even somewhat younger animals than in our study, 17 dpf zebrafish exposed from 0.3 to 30 ng L^{-1} EE2 showed statistically significant induction in a dose-dependent manner in three days [19,52]. Although significant induction of *vtg1* mRNA was observed in very early zebrafish embryos (2 dpf) [50] exposed to 1000 ng EE2 L^{-1}, the same authors also showed significant up-regulation of *vtg1* mRNA in zebrafish embryos exposed to 25 ng L^{-1} for 5 dpf [41].

In *20dpfZF*, *cyp19a1b* was up-regulated in a concentration-dependent manner above 500 µg BPA L^{-1} ($P < 0.05$) and *vtg1* at 1000 µg BPA L^{-1} ($P<0.05$). While in adult fish the induction of Vtg has been documented well [4], post-hatched and early juvenile zebrafish exposed to BPA for 5 d induced *vtg1* at higher concentrations, 2280 µg BPA L^{-1} [41]. Our results showed sensitivity of *20dpfZF* exposed to 1000 µg $BPAL^{-1}$, comparable with adult male zebrafish exposed for three weeks that showed lowest observed effect concentration (LOEC) for vitellogenin protein induction for similar exposure concentration [4]. Thereby, the estrogenic responses of *20dpfZF* present a cost-effective life-stage compared to the adult fish as well as further developed metabolic capacity when compared to embryonic stages.

Unlike BPA, NP showed clear down-regulation of estrogenic response in *20dpfZF*. This is in contrast to significant effects observed in adult [4,53] as well as larval [54] stages of zebrafish and other small bodied fish models, Japanese medaka [55] and fat head minnow [56]. However, other studies this far with zebrafish embryos exposed to NP showed no significant induction of *vtg1* mRNA [36,41], indicating low sensitivity to the chemical. Our results confirmed the low sensitivity of juvenile life-stages to NP as shown by Jin et al. [36]. It also contrasts with the significant induction of aromatase by juvenile zebrafish (17 dpf) exposed to NP for 3d [52]. In our study, the mRNA of both *cyp19a1b* and *vtg1* were down-regulated. In essence, the observed discrepancies can be related to the difference in composition of the selected chemical. Previous studies [4,54] with NP included a technical grade of NP (CAS 84852-15-3); a mixture of isomers. In our study, the linear form of 4-*n*-NP (CAS 104-40-5) that is devoid of branched alkyl side-chains present in most of the isomers, showed slightly reduced estrogenic potency (relative to E2), compared to the higher efficacy of technical grade NP [57]. From metabolite studies of 4-*n*-NP in fish, biotransformation pathway involving β-oxidation of the alkyl side-chain results in metabolites, lacking the alkyl side-chain, which largely accounts for the lesser estrogenic potential of 4-*n*-NP *in vivo* [58].

Among the three alkylphenols in the present study, only OP significantly induced *vtg1* in *20dpfZF*. Earlier studies with adult zebrafish showed the weak estrogenic potential of OP at similar concentrations as used by us [4]. Thus, considering the differences in sensitivity between

life stages, the dose-dependent induction of both *vtg1* and *cyp19a1b* observed in *20dpfZF* is indicative of a suitable window to predict xenoestrogenicity as the key biomarker. It presents a viable and cost-effective life-stage to assess all related chemicals with weak estrogenic potential. In all, from the exposure series with *20dpfZF*, a clear ranking of the estrogenic potency was obtained, with EE2 being the most potent agonist, followed by OP, BPA, and 4-*n*-NP in that order.

Exposure III: *cyp19a1b* and *vtg1* mRNA regulation by sediment spiked with EE2

Gene induction data from the sediment assay with spiked EE2 indicated applicability and distinct sensitivity of the *20dpfZF* in *in vivo* bioassays (Figure 4a and 4b). Due to its physicochemical properties (log K_{ow}=4.15; log K_{oc}=3.8; sorption constant, $1/n$ = 0.83; sorption coefficient, log K_f = 1.72) [42], EE2 was understood to have sorbed to the sediment that consequently acts as a sink for the sediment-water interactions. While the *vtg1* gene expression as shown in Exposure I (Figure 3) indicating the pronounced potency of EE2 even at 50 ng L^{-1} to produce nearly 1400-fold change, a reduced effect was seen in the water-borne exposure to 150 ng EE2 L^{-1}, the positive control (Figure 4b). This can be explained by the static nature of the water-only exposure without renewal of media. Thereby the exposure via water in the static system would result in decline of available EE2 in water phase. Thus the exposure scenario was probably different. In the sediment acting as a sink, thereby partitioning EE2 to water phase to maintain its concentrations towards the sediment-water equilibrium of EE2, was taken up by the fish. Of the two biomarkers, *vtg1* was more sensitive than *cyp19a1b* to sedimental EE2 and indicative even of some influence of electrolyte content in overlying water on the availability of EE2 (Figure 4a and 4b). Mechanistically, as EE2 in Sed 1+ Salt exposure was sorbed to the sediment matrix to a greater extent than in Sed 1, the lesser EE2 content in the aqueous phase consequently elicited a lower induction of *vtg1* (Figure 4b). Considering the same sediment characteristics (TOC %, particle size, spiking, sample preparation) for both samples with the sole exception of overlying water quality (Sed 1+Salt; 0.3% NaCl, 107 mg $CaCO_3$ L^{-1}), this explanation is consistent with studies on the effect of salinity on the higher or lower sorption of EE2 to sediment as shown by Lai et al. [42]. Although, the role of electrolyte content on bioavailability of EE2 can be empirically verified with wide ranges of salinity and hardness levels, this was outside the scope of our study. Moreover, the difference in overlying water was included in our study only to determine variation in biomarker response because E3 salts are generally added in ELS rearing media. While the current trials to assay sediment with *20dpfZF* were first ones of its kind, we did not analyze the dynamics of EE2 content in either compartment. Anyways, in presenting expected responses, the relevancy of *20dpfZF* in sediment bioassay does exist. For further goals, data generated with *20dpfZF* can be employed to study various joint effects of multi-component xenoestrogens.

Choice of suitable developmental stage of zebrafish for evaluation of estrogenicity

Maturity is not the only important life-stage for reproductive success and population maintenance. In support of the relevance of ELS in manifestation of toxicity, age-specific developmental factors, including chorion-permeability and the nutrition by yolk associated with embryonic life as well as post-hatched eleuthero embryos, define important ontogenic stages. There is a common notion that newly post-hatched embryos are the most or equally sensitive life-stage of oviparous fishes to environmental chemicals [19,37,59]. This said, the rule includes several minor exceptions, which in part directed our study to the early post-yolk stages of zebrafish. While several range-finding exposures with various ages of zebrafish to xenoestrogens were examined at large sub-lethal range, a decision was made to focus on *20dpfZF* based on the survivorship as well as consistency of response levels of genomic regulation associated with the life stage. Furthermore, the combined advantages of an ELS that is both free-swimming and free-feeding (Figure 5b), but also cost-effective in terms of husbandry efforts compared to later or adult stages. The life stage was found to be practical and responsive to different media, including here the exposure to EE2-spiked sediment as a contact assay (Figures 5b and 5c).

Regarding xenoestrogenicity of chemicals, the time frame for ontogenic determination of the phenotype in juvenile life can be considered significant. In zebrafish, an undifferentiated gonochorist, ovary-type gonad in the juvenile hermaphrodite stage (21dpf) is transformed into distinct sex-specific mature gonads (42 dpf), leading to phenotypic sex differentiation [60]. At 20-25 dpf, the fish are developmentally more advanced than embryonic stages. However, the possibility of detecting signals from presumptive females from a pool of samples fish is addressed with normalized gene expression quantified relative to control treatments. With as large a sample size (15-25 fish) as in this study, statistically significant difference of estrogenic gene expression between exposures highlights the reliable detection of a genomic signal. Advancement in existing estrogen-sensitive screening has employed the induction of green fluorescent protein expression in transgenic zebrafish, *cyp19a1b-GFP* [44,45] and *ere-zvtg1:gfp* [61], pointing to non-lethal alternatives of animal testing. Chen et al. [61] showed GFP induction in transgenic zebrafish represented by less than 25% of the exposed fish. Sex differentiation in fish, known to be controlled by various environmental as well as of endogenous (steroidogenic) factors, is supported by the role of sex-steroids on differentiation and phenotypic outcome [62]. More specifically, the modulation of aromatase and vitellogenin, both key players in the onset of endogenous estrogen action, can be directly linked to gonadal sex differentiation from endocrine disruptors [63,64]. The choice of a labile life-stage taking into consideration such biological events, directs to the *20dpfZF* as a suitable ELS model for investigating endocrine modulation.

In conclusion, we documented that the post-embryonic (*20dpfZF*) zebrafish was a potent life-stage using transcripts of *cyp19a1b* and *vtg1* as endpoints to assess xenoestrogenicity of chemicals. The RT-PCR presented analytical tools to evaluate xenoestrogenic modulation in a relatively quick approach. The *20dpfZF* life-stage provided for cost-effective maintenance and experimentation (compared to adult fish), and responded to four model estrogens with varying potencies characterized by corresponding induction patterns. The results of this study recommend the selection of *20dpfZF* as a representative life-stage for use in ongoing and further studies, including exposures to water-sediment interactions and mixtures of environmental chemicals.

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Supplementary material

Use of early juvenile zebrafish *Danio rerio* for in vivo assessment of endocrine modulation by xenoestrogens

Contents:

1. Chemical analyses of water samples
2. Tables S1, S2 and S3
3. Figure S1
4. Tables S1 and S2 were created using Word
5. Table S3 and Figure S1 are TIFF files created using CorelDraw (version 12.0)

1. Chemical analysis of water samples

1.1 Solid-phase extraction (SPE) of water samples

Briefly, Oasis SPE cartridges (Oasis HLB, 3cc, 60 mg) were conditioned with 3 mL methyl-*t*-butyl ether (MTBE), subsequently rinsed with 3 mL methanol and 3 mL ultrapure water. Water samples (1000 mL), containing internal standard (d4-EE2), were acidified to pH 3 before loading onto the cartridges. The extraction was managed as a drop-wise outflow, where after the cartridge was re-equilibrated with 3 mL ultrapure water. The elution solvent, 10% MeOH in MTBE (3×2 mL), was added and allowed to react with the analytes on the sorbents and ultimately eluted into collection tubes. The contents were evaporated to dryness while tubes were immersed in a water bath (ca. 50 °C) under a gentle nitrogen flow. Samples were reconstituted in 200 µL 50:50 (v/v) acetonitrile: 2.5 mM ammonium acetate in water (pH 3).

1.2 GC-MS analysis of EE2

The dried residues were treated (silylated) with 50 µL BSTFA (+1%TMCS) and 50 µL pyridine, incubated at 70 °C for 30 min, where after cooled and evaporated to dryness with nitrogen stream. Finally, hexane (100 µL) was added, followed by transferring the contents to auto sampler vials for the GC–MS, performed with HP 6890 gas chromatograph (Hewlett-Packard, Walbronn, Germany) equipped with HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, CA, USA). A HP-5 capillary column (30 m × 0.25 mm × 0.25 µm) was used with helium as the carrier gas by a rate of 0.9 mL min^{-1}. The column temperature was programmed to increase from 120 °C to 190 °C at the rate of 8 °C min^{-1}, 190 °C to 240 °C at 2 °C min^{-1}, 240 °C to 280 °C at 10 °C min^{-1} (maintained for 8 min), and 280 °C to 290 °C at 10 °C min^{-1} (maintained for 20 min). The MS was operated in selective ion monitoring mode, and identification was made based on retention time and mass spectra of pure standards.

1.3 LC-MS /MS analysis of BPA, NP and OP

Separation with a reverse phase C18 column (Waters XBridge 3.5 µm, 2.1×100 mm with 3.5 µm,2.1×10 mm guard column) was performed with Waters Alliance 2795 (MA, USA) LC consisting of tertiary pump, vacuum degasser, auto sampler and column oven. The column temperature was set to 30 °C and that of auto sampler to 20 °C. The injection volume was 20 µL. The determination of target compounds was performed in negative ion electrospray mode (ESI-) and a Quattro Micro triple quadrupole mass spectrometer (MS/MS) (Waters, MA, USA) with

electrospray interface was used as detector. The mobile phases consisted of MeOH (A) and distilled water (B) with a flow of 0.2 mL min^{-1}. The gradient of A was raised to 100% over 10 min, phase proportion maintained for 8 min (10-18 min), and lowered back to 60 % over 2 min. The column was equilibrated for three min (20-23 min) prior to the next injection.

Compound	Fw	Precursor ion m/z	Product ions m/z	Cone voltage (V)	Collision energy (eV)
BPA	228.3	227.1	133.1, 212.0	39	21
d16-BPA(IS)	244.06	241.1	142.1, 223.1	41	28
NP	220.4	219.3	106.0, 118.7	40	34
OP	206.3	205	133	38	27

Table S1: Acquisition parameters of mass spectrometry for target compounds and corresponding internal standard (IS; d16-BPA) used in this study.

Gene	Primer sequences (5'-3')	Size (bp)	Genbank accession
β-actin	Forward-AAGAGCTATGAGCTGCCTGA Reverse-ACCGCAAGATTCCATACCCA	108	AF057040.1
cypl9alb	Forward-TGGACGCATGCATAAGACAG Reverse-CACAACCGAATGGCTGGAA	104	AF226619.1
efla	Forward-AAACATGGGCTGGTTCAAGG Reverse-ATGGCATCAAGGGCATCAAG	87	AY422992.1
cypllal	Forward-GCCTGGAAGACTGATAGGATGA Reverse-GTTGACACGAGCCACAAAGT	114	AF527755.1
star	Forward-TGTAAGGGCTGAGAATGG Reverse-TACTCGGTTGATGACAGTT	133	NM_131663.1
vtgl	Forward-GCTTTGCCTATTCCCACATC Reverse-GCTCTGCTGTAACGGTAGT	136	NM_001044897.2
hsd3bl	Forward-AGCCCATTCTGCCCATCTTT Reverse-CCATGGTCACTAGCTGCCTATT	120	AY279108.1

Table S2: Primer pair sequences, amplicon sizes, and accession numbers for genes investigated in this study (*β-actin*, beta-actin; *ef1a*, elongation factor 1a; *vtg1*, vitellogenin isoform 1; *cyp19a1b*, brain aromatase; *star*, steroidogenic acute regulatory protein; *cyp11a1*, P450 side chain cleavage; *hsd3b1*, 3-beta-hydroxysteroid dehydrogenase). Note the primer design for VTG1 was custom ordered from Sigma-Aldrich.

EE2(ng L^{-1})			BPA (µg L^{-1})			OP (µg L^{-1})		
Nominal	Measured	% Nominal	Nominal	Measured	% Nominal	Nominal	Measured	% Nominal
5	1.7 ± 0.6	34	100	92 ± 11	92	10	3.9 ± 1.2	39
25	10.8 ± 0.1	43	500	410 ± 40	82	50	20.2 ± 8.0	40
50	28.3 ± 6.4	57	1000	808 ± 307	81	100	45.2 ± 8.7	45

Table S3: Nominal and measured concentrations of EE2, BPA and OP in exposure series I and II. Measured concentrations (mean ± sd) were determined for exposure samples collected at two time points (days 1 and 4) along the five-day exposure period.

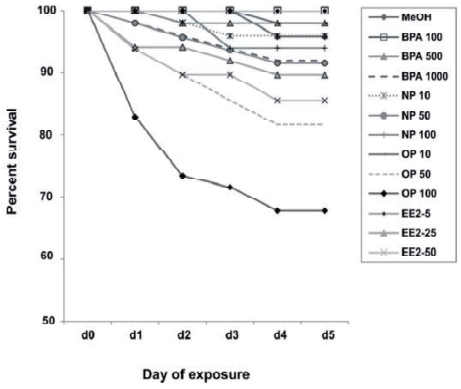

Figure S1: Survival plot of *20dpfZF* exposed to xenoestrogens in Exposure I. The data represent mean of three replicates per experimental and control groups each containing n = 25 fish. Note experimental groups exposed to OP (50 and 100 µg L^{-1}) and EE2 (50 ng L^{-1}) showed mortality (%) at 18, 32 and 15 respectively.

II

Modulation of estrogen-responsive gene induction by xenoestrogenic mixtures in early juvenile zebrafish.

by

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Tuula Heinonen & Aimo Oikari

Submitted manuscript

III

Use of early life-stages of zebrafish to assess toxicity of sediments contaminated by organotin compounds

by

Tarini Prasad Sahoo & Aimo Oikari

Submitted manuscript

IV

Probing the xenoestrogenic impact of sediment cores contaminated by the pulp and paper industry: Induction of aromatase *cyp19a1b* in early juvenile zebrafish.

by

Tarini Prasad Sahoo & Aimo Oikari

Submitted manuscript