Effect studies of real life mixtures of persistant organic pollutants (POPs) in a zebrafish model

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Contents

Summary

The release of persistent organic pollutants (POPs) into the environment for many decades has led to a worldwide contamination. The impact of pollutants on ecosystem and human health is an urgent and international issue since there are an increasing number of examples of environmental disturbances as well as documented detrimental effects in humans and wildlife. The potential of POPs to cause toxicity in the reproductive and endocrine systems, as well as other adverse effects, has been of growing concern in the Western world. Despite the fact that individuals are more likely to be exposed to combinations of environmental pollutants, many experimental studies have focused on individual compounds or commercial mixtures of POPs.

The present study focus on effects of real life mixtures of POPs harvested from aquatic ecosystems in Norway in an *in vivo* zebrafish model. POPs were extracted from burbot liver from Lake Losna and Lake Mjøsa and were used to expose the zebrafish through their diet in a two-generation study. Genome-wide effects on gene expression, biomarker detection, together with whole-organism responses, such as survival, growth, sex ratio and onset of puberty were investigated.

The concentration range of POPs measured in zebrafish was lower than in burbot originating from Lake Mjøsa, but comparable to concentrations previously reported in humans and wildlife.

The results showed that exposure of zebrafish with real life mixture of POPs had a negative effect on survival of fish in both generations. The marked drop in survival during 9-20 dpf suggested that this period may be a critical window of development in the zebrafish. In the parent (F0) generation there was a higher proportion of males and an earlier onset of puberty in Losna and Mjøsa exposed groups. Exposure with Losna and Mjøsa mixtures increased body mass and length in the parent generation (F0), while in the next generation (F1) the same exposures were associated with a decrease in body mass and length.

Additionally, an indirect transgenerational effect of onset of puberty was indicated by the fact that exposure in F0 (Losna and Mjøsa High) combined with similar exposure in F1 resulted in an earlier onset of puberty in F1 (Losna-Losna and Mjøsa High-Mjøsa High). In terms of sex ratio, a possible transgenerational effect of Mjøsa and Losna exposure was also suggested by the fact that the groups receiving these exposures in F0 maintained a sex ratio skewed towards male dominance, whereas groups exposed to the same mixtures in F1 did not.

Subtle differences were detected in mRNA levels, and altered gene expression was only found for the Mjøsa High exposure group. Genes associated with a cellular stress response, apoptosis, AhR signalling, and endocrine regulation was found to react to the toxic challenge. The biomarkers used in the present study (7-ethoxyresorufin-*O*-deethylase, EROD; Vitellogenin, Vtg) did not reveal strong dioxin-like effects or oestrogenic effects, respectively, that could be associated with exposure with real life mixtures of POPs.

It was concluded that long term and low level exposure to real life mixtures of POPs was associated with effects linked to survival, development and reproduction.

1. Abbreviations

AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
AR	Androgen receptor
ARG	Arginase
ARNT	AhR nuclear translocator
CALCOCO1	Calcium binding and coiled-coil domain 1
CALUX	Chemical activated luciferase gene expression
cAMP	Cyclic adenosine monophosphate
CHX	Cyclohexane
CNS	Central nervous system
COX	Cytochrome c oxidase
Cy3	Cyanine 3
Cy5	Cyanine 5
CYP	Cytochrome P450
DAVID	Database for annotation, visualization and integrated discovery
DDD	Dichloro-diphenyl-dichloroethane
DDE	Dichloro-diphenyl-dichloroethylene
DDT	Dichloro-diphenyl-trichloroethane
DRE	Dioxin response elements
DMSO	Dimethyl sulfoxide
Dpf	Days post fertilization
DUSP1	Dual specificity phosphatase 1
E2	17β-estradiol
EE2	17α-ethynylestradiol
EDC	Endocrine disruption compound

EDF1	Endothelial differentiation-related factor 1
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ER	Oestrogen receptor
EROD	7-ethoxyresorufin-*O*-deethylase
EU	European Union
FDR	False discovery rate
FSH	Follicle-stimulating hormone
GABA	Gamma-amino butyric acid
GnRH	Gonadotropin-releasing hormone
GPM	Gallons per minute
HBCD	Hexabromocyclododecane
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HPG	Hypothalamic-pituitary-gonadal
Hsp	Heat shock proteins
HSDL2	Hydroxysteroid dehydrogenase like 2
iDREs	Inhibitory dioxin-responsive elements
IVT	*In vitro* transcription
IPA	Ingenuity pathways analysis
LH	Luteinizing hormone
LimmaGUI	Linear models for microarray data graphical user interface
Loess	Locally weighted scatterplot smoothing
MDH2	Malate dehydrogenase 2
NCOA4	Nuclear receptor coactivator 4
NIVA	Norwegian institute for water research
NLS	Nuclear localization sequence

NPM1	Nucleophosmin 1
OC	Organochlorine
OH-PBDE	Hydroxylated polybrominated diphenylether
OH-PCB	Hydroxylated polychlorinated biphenyl
PBDE	Polybrominated diphenylether
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PDCD10	Programmed cell death 10
POP	Persistent organic pollutant
RIN	RNA integrity number
RPL	Ribosomal protein like
Rpm	Rounds per minute
SA	Sulphuric acid
SE	Standard error
SEC24D	Sec24 related protein D
SERP1	Stress-associated endoplasmic reticulum protein 1
SLC25A5	Solute carrier family 25 member 5
SMNDC1	Survival motor neuron domain containing 1
SSR1	Signal-sequence receptor α
TCDD	2,3,7,8-tetradibenzo-*p*-dioxin
T3	Triiodothyronine
T4	Thyroxine
TEF	Toxic equivalence factor
TTR	Transtyrethrin
UPR	Unfolded protein response
Vtg	Vitellogenin
WHO	World health organization

XRE	Xenobiotic response elements

2. Introduction

2.1 Worldwide chemical pollution

Since the 1930's persistent organic pollutants (POPs) has been released into the environment in Europe, as well as other industrialized countries. This release is a consequence of agricultural productivity and a result of modern manufacturing processes and their by-products in the industry. The lipophilic nature and their resistance to degradation cause POPs to biomagnify in food chains, allowing concentrations of these compounds to increase as they pass through each trophic level, and consequently resulting in high POP levels in adipose tissue in top predators (Corsolini et al., 2007;Corsolini et al., 2002). Alarming implications of this is the contamination of foodstuff, which has led to recommendations of restricted consumption of marine and freshwater products in various regions.

Concerns about possible detrimental effects in the environment, humans and wildlife have been raised. A major focus has been on POPs potential to act as endocrine disruptors by mimicking endogenous hormones as well as interfering with hormone transport proteins in plasma (Layton et al., 2002;Meerts et al., 2001;Ulbrich and Stahlmann, 2004). Furthermore, reported effects, such as reproductive impairment, neurotoxicity, immune suppression and carcinogenicity giving rise to potential health hazard are alarming (reviewed in Darnerud et al., 2001;Mandal, 2005;Turusov et al., 2002;Ulbrich and Stahlmann, 2004).

The discovery of POPs in the Arctic, far away from their original source, indicates long-range transport with sea currents and in the atmosphere, and that these compounds have the potential for global contamination (Ikonomou et al., 2002;Muir and Norstrom, 2000). Consequently, the widespread use and production of POPs has led to a ubiquitous presence; a problem with great relevance to wildlife,

environmental and human health. Although many contaminants have been banned and their use restricted, there is still an ongoing pollution crises.

2.2 The pollutants

The POPs described in this section belong to the group of halogenated organic compounds consisting of a backbone of hydrocarbons, where hydrogen to a varying degree is substituted with halogens (bromine or chlorine). In the present study, the described POPs do not occur alone, but they are included in mixtures harvested from natural ecosystems and used in exposure experiments.

Brominated flame retardants (BFRs)

The BFRs has been used in a variety of commercial and household products, especially plastics, textiles and electronic equipment and circuitry, to prevent fire-related injury. These toxins are numerous and vary in structure and physiochemical properties. However, they all contain the bromine atom that is released with heat and quench the radical cascade of the fire-spreading process (Hooper and McDonald, 2000). Some of the BFRs are additives mixed into polymers and are not chemically bound to the products and therefore may separate and leach into the environment (Law et al., 2006;de Wit, 2002;Ikonomou et al., 2002).

Two major groups of BFRs are the polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD). The general structure of these components is shown in Fig. 2.1. PBDEs are structurally similar to the PCBs with the same nomenclature and number of congeners (209), which are divided into 10 groups; mono- to deca-PBDEs (Siddiqi et al., 2003).

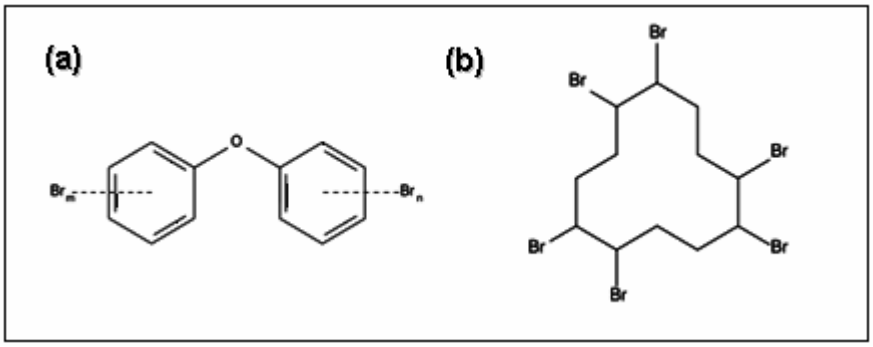


Figure 2.1: *Chemical structure of (a) polybrominated diphenyl ethers (PBDEs) and (b) hexabromocyclododecane (HBCD). Figure from http://en.wikipedia.org/wiki/.*

PBDEs

Commercial mixtures of PBDEs have been produced and used since the 1960's as additives to retard fire. The carbon-bromine bond in PBDEs is weaker compared to the carbon-chlorine bond in PCBs, indicating that the bromine atom being released upon heating is a better "leaving group" than chlorine, and thus are more thermal stabile (Hooper and McDonald, 2000). They are manufactured by bromination of diphenyl ethers under conditions that result in mixtures of PBDEs, and commercial mixtures consist mainly of penta-, hepta-, octa-, and decabromodiphenyl ethers (Darnerud et al., 2001). Despite banning and restricted use of PBDEs, levels of these contaminants are still increasing (Meironyte et al., 1999). The European Union (EU) banned the use of penta- and octa-PBDE by mid-2004 and restricted the use of deca-PBDE by mid-2006 (Madsen et al., 2003). California also took action to a restricted use of PBDEs (California State Senate, 2003). According to a breast milk monitoring programme in Sweden, they have forced manufactures to reduce their PBDEs levels (California State Senate, 2003;Hooper and McDonald, 2000;Madsen et al., 2003).

The first evidence of PBDEs in the biota was found in fish in Sweden (Andersson and Blomkvist, 1981), and later these pollutants were detected in air, soil, sediments, wildlife, aquatic organisms and sewage sludge (Darnerud, 2003;Law et al., 2006;de Wit, 2002). Additionally, they have been found in human blood, adipose tissue and in

breast milk (Hooper and McDonald, 2000;Darnerud et al., 2001;Meironyte et al., 1999). The major congeners in human tissues and in marine and terrestrial life are the three *ortho-para*-substituted congeners; 2,2',4,4'-tetra-BDE (BDE-47), 2,2',4,4',5-penta-BDE (BDE-99), 2,2',4,4',6-penta-BDE (BDE-100) and 2,2',4,4',5,5'-hexa-BDE (BDE-153), whilst deca-BDE seems to be less prevalent because of its low bioavailability (Hooper and McDonald, 2000;Darnerud et al., 2001;Meironyte et al., 1999;Rice et al., 2002). However, in Japan, BDE- 209 is the most prevalent congener detected in human and animal samples (unpublished data), most probably because its use is still allowed whereas the use of lower brominated congeners, such as tetra- and octa-BDE, has not been recognized after 1991 and 2000, respectively (Watanabe and Sakai, 2003).

The structural similarity PBDEs share with PCBs indicates that some of their toxicological properties appear similar. Their toxicity might also be due to hydroxylated metabolites (OH-PBDEs), which may have more severe biological effects (Meerts et al., 2001). Evidence of PBDEs acting as endocrine disruptors has been shown for some congeners and OH-metabolites *in vitro* (Meerts et al., 2001;Nakari and Pessala, 2005). Also they have been shown to cause thyroid dysfunction in rats (Hallgren and Darnerud, 2002;Darnerud et al., 2007;Zhou et al., 2001).

Furthermore, reported effects include also neurobehavioral alterations (Eriksson et al., 2002), reproductive disorders (Kuriyama et al., 2005) and activation of liver enzymes, like 7-ethoxyresorufin-*O*-deethylase (EROD) through the aryl hydrocarbon receptor (AhR) mediated pathway (Zhou et al., 2001;Nakari and Pessala, 2005). Deca-BDE was reported to cause liver tumours in rats at relatively high doses (NTP, 1986).

Radiolabelled BDE-47 has been found to bind covalently to macromolecules and lipids in rats and mice, suggesting a genotoxic mechanism (Orn and Klasson-Wehler, 1998). In addition, thermolysis and photolysis of PBDEs may give rise to hazardous

compounds such as polybrominated dibenzo-p-dioxin (PBDD) and polybrominated dibenzofuran (PBDF) (Buser, 1986).

HBCD

Since the 1980's HBCD has been used as an additive flame retardant mostly in textiles and articles made of polystyrene. The physical and chemical properties of this compound shows, like all other POPs, a lipophilic nature and resistance to degradation (KEMI, 2006), indicating an affinity for particles and thus has the ability for atmospheric transport. Commercial HBCD consists of three isomers: α-, β-, and γ-HBCD, and their content varies depending on application (Birnbaum and Staskal, 2004). The use of HBCD has no restrictions and there has been a trend towards replacing PBDEs by HBCD in Europe, except in Sweden where HBCD is not used in the textile industry (Remberger et al., 2004).

Information about HBCD toxicology is incomplete, however HBCD was detected in air, sewage sludge, sediments, and fish (Sellstrom et al., 1998;Remberger et al., 2004). Reports suggest that this pollutant can disrupt behaviour, learning and memory skills (Eriksson et al., 2006). In addition, decreased thyroid hormone levels has also been shown (van der Ven et al., 2006). Furthermore, a recent study showed that HBCD can reduce uptake of dopamine and to some degree glutamate uptake suggesting its potential to cause neurobehavioral disruption (Mariussen and Fonnum, 2003).

Polychlorinated biphenyls (PCBs)

PCBs are among the first known POPs. They have been used since the 1930's in industrial applications as cooling and insulating fluids for transformers and capacitors, and as additives in plastics and as surface coatings due to their chemical and physical stability and low flammability. Manufacturers produced PCBs by catalytic chlorination of biphenyls resulting in mixtures of multiple isomers with different degrees of chlorination (Fig. 2.2). They were mass produced and globally

distributed through use and disposal long before their detrimental effects were known. After evidence of bioaccumulation of PCBs in food chains in Sweden and United States, together with an accidental poisoning in Japan in the 1960s, PCB production was banned by the U.S. Congress in 1976 (Madsen et al., 2003). Levels have declined, but still PCBs are among the most predominated POPs in the environment and in food chains.

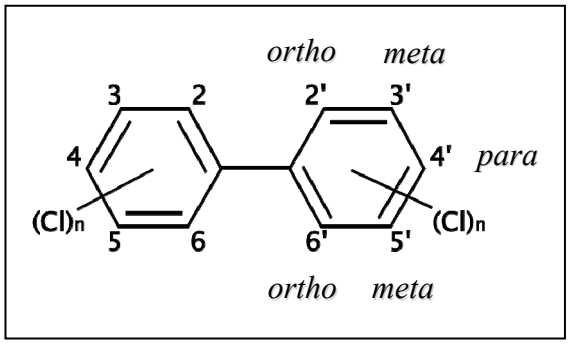


Figure 2.2: *Chemical structure of the PCB molecule. It consists of ten positions where chlorine can be substituted in ortho, meta and/or para positions of the biphenyl backbone; mono- to deca-PCB. Different chlorination patterns and degrees of chlorination give rise to 209 possible congeners. Figure from http://en.wikipedia.org/wiki/.*

Theoretically 209 PCB congeners are known, and their toxicity and lipophilicity vary. Higher chlorination pattern increases the lipophilicity and bioaccumulative properties of the PCBs. They can be divided into three classes based on their substitution of chlorine atoms in *ortho*-position; non-*ortho,* mono-*ortho* and di-*ortho* PCBs.

The non-*ortho* substituted (coplanar, dioxin-like) PCBs (i.e. PCB-77, PCB-126, and PCB-169) which have no chlorine atoms in *ortho* position, and a few mono-*ortho* PCBs (i.e. PCB-118), exist in nature at relatively low levels. They are regarded as the most toxic PCBs due to their dioxin-like properties by eliciting their mechanistic effect through the AhR pathway (Section 2.4). The toxic responses by this pathway

include induction of biotransformation enzymes, such as CYP1A1 and CYP1A2, endocrine effects, developmental toxicity, hepatoxicity, immunotoxicity and decreased vitamin A levels (reviewed in Safe, 1994). Decreased thyroxin (T4) levels have been observed in rodents exposed to PCB-77 and PCB-169 due to their ability to induce UDP-glucoronosyltransferases, which conjugate triiodothyronine (T3) and T4 (Morse et al., 1993). Furthermore, PCB-77 has been shown to reduce the number of germ cells in the ovaries by 40 - 50 % suggesting premature reproductive aging (Ronnback, 1991). However, this reduction did not lead to a significant change in the reproductive capacity during the test period.

The remaining PCBs, the di-*ortho* substituted, which have two or more chlorine atoms in the ortho positions, occur in nature at high levels. PCB-138, PCB-153 and PCB-180 are the dominating congeners in human adipose tissue and breast milk, where PCB-138 and -153 account for 40 -60 % of the reported PCBs (Hansen, 1998). These PCB congeners seem not to activate the AhR and exert their effects via other mechanisms. Several studies have demonstrated interference with the endocrine system such as reduced thyroid hormone levels *in vitro* due to specific binding to transtyrethrin (TTR), a thyroxin transport protein (Chauhan et al., 2000). Lower levels of T4 caused by PCBs have also been identified in humans (Persky et al., 2001). Moreover, PCB-38, -153 and -180 have shown an affinity to oestrogen receptor (ER) leading to an antiestrogenic effect (Bonefeld-Jorgensen et al., 2001). In the same study, PCB-138 was also found to cause a dose-dependent antagonistic effect on the androgen receptor (AR). Low doses of PCB-153 has been shown to delay the onset of puberty in goats exposed from day 60 in gestation and during suckling (Lyche et al., 2004b). Additionally, the same exposure was reported to cause immunotoxic effects (Lyche et al., 2004a).

The di-*ortho* substituted congeners have been shown to interfere with calcium dependent intracellular signalling pathways suggesting a possible role in neurotoxicity (Simon et al., 2007).

Metabolism of PCBs to form hydroxyl- or methylsulfonyl metabolites, gives rise to more easily excreted compounds which have also been identified as endocrine disruptors. Hydroxylated PCBs (OH-PCBs) have binding affinities for oestrogen receptors at environmentally relevant concentrations which may lead to antiestrogenic as well as estrogenic activity (Kramer et al., 1997;Layton et al., 2002). Methylsulfonyl metabolites of PCBs have shown to reduce T4 and T3 levels in serum in adult rats (Kato et al., 1999).

Reproductive disorders have also been associated with PCBs. A field study with harbour seals fed environmentally contaminated herring showed a causal relationship between the natural occurring PCBs and the reproductive response (Reijnders, 1986).

Dioxins

The term “dioxins” usually includes polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzo-furans (PCDFs). PCDDs and PCDFs have never been commercially manufactured but are mainly by-products from industrial processes, such as metallurgical processing, combustion of municipal waste, chlorine bleaching of paper and production of some herbicides and pesticides. Natural processes like wood fires and volcanic eruptions may also contribute to PCDD and PCDF release into the environment. Today, dioxins are found in humans and food products, with higher concentrations near industrialized regions (Schecter et al., 2003b;Schecter et al., 2001;Baars et al., 2004).

The lipophilic nature and slow degradation of dioxins leads to accumulation in body tissues with high lipid content. Their toxicity varies depending on the chlorination pattern and degree of chlorination. 2,3,7,8-tetradibenzo-*p*-dioxin (TCDD; Fig. 2.3) is the most toxic compound known. Dioxin congeners, including the coplanar (dioxin-like) PCBs, show the same type of toxicity mainly through the AhR pathway (Section 2.4). Based on the ability of dioxins to activate AhR, a concept of toxic equivalency factors (TEFs) has been developed in order to evaluate their toxicity relative to the

most toxic congener TCDD, which has been given the TEF value 1 (Van den et al., 1998;Safe, 1994).

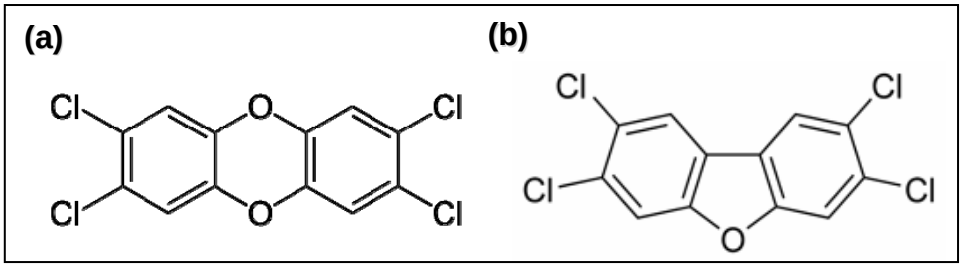


Figure 2.3: *Chemical structure of (a) 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and (b) 2,3,7,8-dibenzofuran (TCDF). Figure from http://en.wikipedia.org/wiki/.*

A wide variety of toxic effects of these compounds have been demonstrated, where the toxicity is mediated mainly through the AhR, which is responsible for upregulation of biotransformation enzymes. In addition, dioxin-activated AhR was found to cause endocrine disruption by antiestrogenic action (Hruska and Olson, 1989). Identification of inhibitory dioxin response elements (iDREs) in promoter regions of ER regulated genes demonstrated that several oestrogen-induced genes are inhibited by dioxins. An *in vitro* study showed a depletion of 17β-estradiol (E2) caused by ER degradation was related to AhR agonists (Safe, 2001). The reproductive toxicity of dioxins has been well studied and perinatal exposure in rodents appeared to reduce the reproductive potential of female, along with a reduced sperm production in male progeny (Mandal, 2005;Peterson et al., 1993). Other studies have reported impaired reproductive success in zebrafish after exposure to TCDD for 4 weeks (Heiden et al., 2005;Heiden et al., 2006).

TCDD is highly immunosuppressive and affect the immune system in several ways, additionally dioxin promotes inflammatory responses due to an upregulation of inflammatory cytokines (Spitsbergen et al., 1986;Holladay, 1999;Kerkvliet, 1995).

Furthermore, TCDD has been found to cause neurodevelopment defects in embryonic life stages in the zebrafish (Hill et al., 2003). The carcinogenicity of TCDD has also

been studied, and found to be a tumour promoter (Grinwis et al., 2000), and increase incidences of neoplasm in liver, lung and oral mucosa (Walker et al., 2006). Additionally, TCDD has proved to be liver toxic in zebrafish (Zodrow et al., 2004).

Organochlorine pesticides

The organochlorine pesticides is a diverse group of compounds, which can be divided into three classes based on their chemical structure; dichlorodiphenylethanes (i.e. DDTs), cyclodienes (i.e. chlordanes) and chlorinated benzene- and cyclohexane-related structures [Hexachlorobenzene (HCB), hexachlorocyclohexane (HCH); (Casarett and Doull, 2001)].

DDT

Dichloro-diphenyl-trichloroethane (DDT; Fig. 2.4) is a long-lived POP. It was the first widely used organochlorine insecticide and revolutionised agricultural production, and prevented people being infected with insect-borne diseases, mainly malaria. Even though the benefits of the widespread use of this pesticide were great, DDT is regarded as a hazard to the environment due to its chemical stability, slow degradation and bioaccumulation in adipose tissues. Commercial DDT is a mixture primarily consisting of *p*-*p*-DDT and *o*-*p*-DDT, and is broken down in the environment to mainly dichloro-diphenyl-dichloroethylene (DDE) and dichloro-diphenyl-dichloroethane (DDD; Fig. 2.4) which are persistent.

The use of this pesticide started in the 1930's and resulted in a global spread of DDT. In the 1970's and 1980's DDT was banned in most developed countries, while in tropical areas it is still used as a vector control (WHO, 1979). The worldwide pollution of this chemical has decreased dramatically, however exposure is still unavoidable. DDT can be transported over long distances by air and sea currents, and has been detected in water melted from Antarctic snow and in soil not treated with the pesticide. Furthermore, this agent has also been found in a variety of food products, particularly with animal origin, like milk, meat, cheese, as well as in oil,

vegetables, spices and cereals (WHO, 1979;Kaphalia et al., 1990). Thus human exposure to DDT is likely to come from contaminated food.

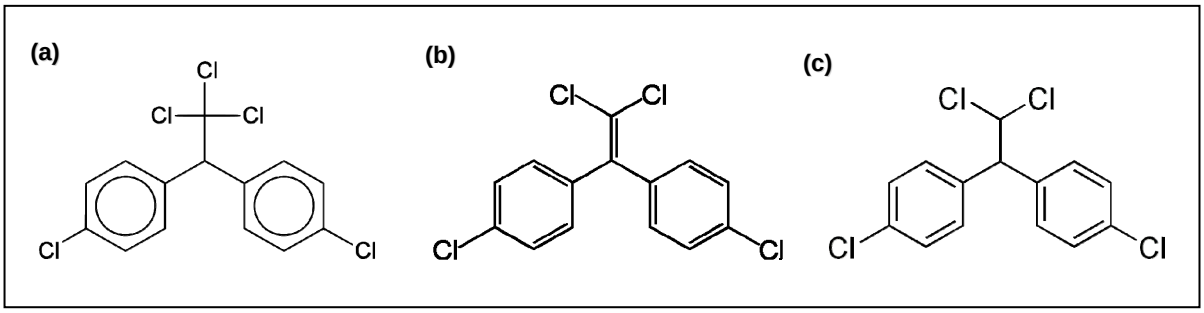


Figure 2.4: *Chemical structure of (a) DDT, (b) DDE and (c) DDD. Figure from http://en.wikipedia.org/wiki/.*

DDT act as an insecticide by interfering with sodium-potassium pumps in the central nervous system (CNS) of insects thereby disturbing the conductance of nerve impulses (Casarett and Doull, 2001). DDT exposure was also found to cause neurotoxicity in other animals suggesting a similar mechanism of action (Bornman et al., 2007). One of the first indications that DDT could have a negative impact on wildlife was the decrease in eggshell thickness in British birds after DDTs introduction (Ratcliffe, 1970). Moreover DDT was detected in humans, particularly in breast milk of nursing women worldwide, which obviously raised concerns about suckling babies (Smith, 1999). A Canadian study suggested that prenatal DDT exposure increased the risk of infectious diseases in children (Dewailly et al., 2000).

In vitro studies indicate that DDT and its DDE metabolite might act as endocrine disruptors; DDT was shown to have estrogenic activity (Chen et al., 1997) and DDE may work as an androgen receptor antagonist (Kelce et al., 1995). In addition, estrogenic effects of DDT have been shown in medaka fish, where females underwent a functional sex-reversal (Edmunds et al., 2000). Furthermore, reports on effects of occupational DDT exposure has demonstrated an increased risk for liver

cancer (Cocco et al., 1997) as well as reproductive disorders (Longnecker et al., 2001). As with other organochlorine chemicals, DDT has the ability to induce CYP enzymes, and subsequent reactive products may form (Turusov et al., 2002).

Chlordane

Chlordane (Fig. 2.5) is a pesticide used on crops, like corn and citrus, and in gardens as well as combating termites. It is manufactured as a mixture of pure chlordane together with many related chemicals. Due to harm in the environment and human health, the United States Environmental Protection Agency (EPA) banned all use of chlordane in 1983, except for termite control (ATSDR, 1995).

Chlordane acts as a CNS poison by antagonize the action of the GABA (gamma-amino butyric acid) receptor (Casarett and Doull, 2001). This agent seems to have adverse effect in the nervous system and digestive system, as well as causing liver problems (ATSDR, 1995;Casarett and Doull, 2001).

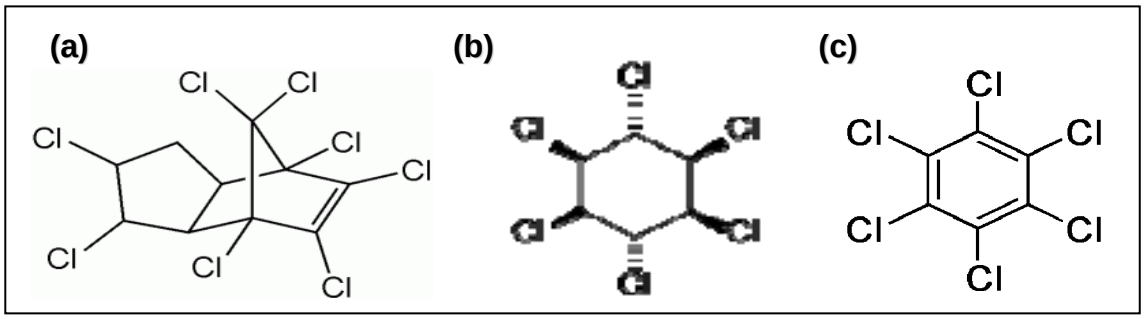


Figure 2.5: *Chemical structure of (a) chlordane, (b) HCB and (c) γ-HCH. Figure from http://en.wikipedia.org/wiki/.*

HCB

Since the 1940's hexachlorobenzene (HCB; Fig. 2.5) have been used as a fungicide for seed treatment, especially on wheat. However, from 1965, its subsequent use was restricted. HCB is a by-product from production of other chemicals, and it may also

be synthesized during combustion of municipal waste. This compound has shown a slow degradation potential and ability to bioaccumulate in tissues with high lipid content.

HCB has been detected in air, water, food products and in humans (WHO, 2004). Studies in animals demonstrate that this agent can cause disturbances in different organs, such as liver, kidney and spleen, and disturb the immune system and the endocrine systems (ATSDR, 2002;Casarett and Doull, 2001;WHO, 2004).

HCHs

Hexachlorocyclohexanes (HCHs) has been used as an insecticide for seed treatment for the past 50 years. They are manufactured by chlorination of benzene resulting in a mixture of isomers, α-HCH, β-HCH, γ-HCH, δ-HCH and ε-HCH, where α-, β- and γ-HCH are the most common isomers found in the environment (POPRC, 2006). They all cause disturbances in the CNS, where γ-HCH and α-HCH act as convulsants, whereas β-HCH and δ-HCH are depressants (Casarett and Doull, 2001). γ-HCH, also known as lindane (Fig. 2.5), has been used in shampoos and lotion to control head lice and scabies as well as for agricultural purposes (POPRC, 2006). The use of lindane for seed treatment has been strongly restricted and currently it is banned in 52 countries, while it use is still permitted in pharmaceutical products (PANUPS, 2006).

Lindane and its isomers are toxic to humans, animals and the environment, and are suspected to be carcinogens and hormone disruptors. A significant association between brain tumours in children and use of lice shampoo containing lindane has been reported (PANUPS, 2006).

2.3 Endocrine disruption

Pollutants interfering with the endocrine system, known as endocrine disrupting compounds (EDCs), elicit their effects by a variety of mechanisms by influencing normal reproduction and development in an intact organism or its progeny. These

processes are strictly coordinated by hormonal signals in order to maintain homeostasis for avoiding wide fluctuations in hormone levels or responses. EDCs are widely distributed in the environment, and numerous compounds have been identified as endocrine disruptors, ranging from natural (i.e. E2), synthetical (i.e. EE2, 17α-ethynylestradiol), pesticidal (i.e. DDT and its derivatives) to industrial compounds (PCBs, PBDEs and their derivatives) (Colborn et al., 1993;Meerts et al., 2001;Mills and Chichester, 2005). In this thesis, the main focus is on the influence of POPs on reproduction and development.

HPG axis

The reproductive system is highly coordinated primarily by the HPG (hypothalamic-pituitary-gonadal) axis which takes place mainly during foetal and neonatal periods (WHO, 2002). This description is somewhat simplified compared to the *in vivo* context and a schematic model of the system is presented in Fig. 2.6.

Upon environmental signals such as temperature and photoperiod integration through the brain, GnRH (gonadotropin releasing hormone) is released from GnRH neurons projecting from the hypothalamus and binds to receptors in the anterior pituitary gland. This stimulates the secretion of gonadotropic hormones (LH, luteinising hormone and FSH, follicle-stimulating hormone) of the anterior pituitary gland, which act on their respective target cells in the gonads (LH on theca/Leydig cells; FSH on granulosa/Sertoli cells). An increase of gonadal sex steroids (E2 mainly in the female and testosterone mainly in the male) in the bloodstream induces the expression of sex steroid responsive genes, making a change in sexual characteristics in an organism. In order to maintain homeostasis, a negative feedback is then transmitted to the hypothalamus and pituitary gonadotropes to reduce the levels of GnRH, LH and FSH (WHO, 2002).

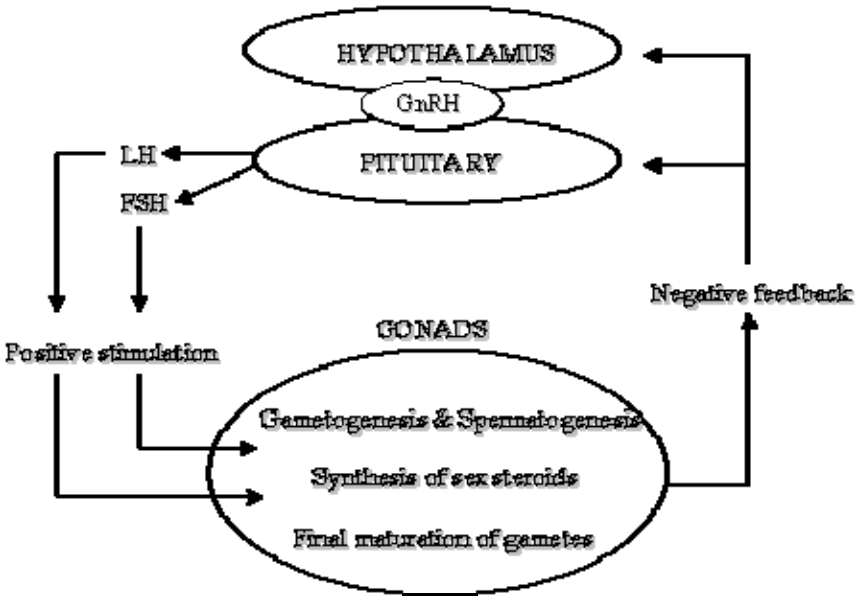


Figure 2.6: *Diagrammatic representation of the main parts in the hypothalamus-pituitary-gonadal axis. GnRH is secreted from the hypothalamus and transported in blood to the pituitary where it stimulates synthesis and secretion of LH and FSH. Target cells in the gonads are triggered by LH and FSH to synthesise sex steroids, which in turn have a negative feedback on the synthesis and secretion of GnRH, LH and FSH.*

Sex steroid biosynthesis

Synthesis of sex steroids from cholesterol, mainly in gonads and adrenal glands, requires an array of enzymatic steps in mitochondria and in the endoplasmatic reticulum. Cholesterol is converted to pregnolenone by CYP11A1, which itself is not a hormone but the precursor for all steroid hormones. Pregnolenone is further converted in many steps which subsequently giving rise to the biologically active sex steroids testosterone and estradiol. Binding of E2 to the ER and testosterone to the AR, consequently initiates transcription of target genes by binding to response elements in regulatory gene regions (Whitehead and Rice, 2006). Detailed overview of the steroidogenesis, including pathways and enzymes, is presented in Fig. 2.7.

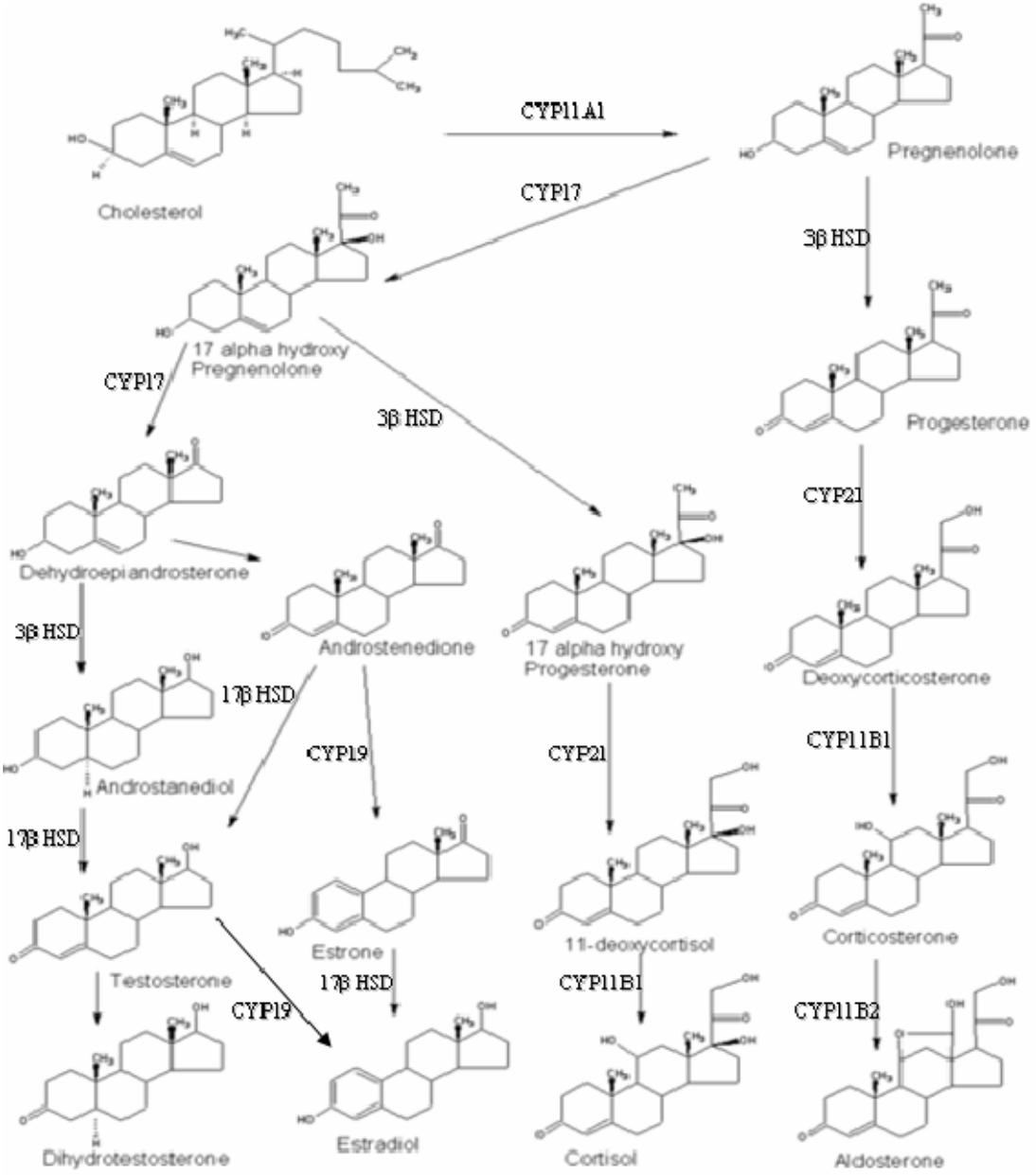


Figure 2.7: *Steroid synthesis of the sex hormones and the enzymes required for the synthesis of the major classes of steroids. The conversion of testosterone to estradiol is catalysed by CYP19, also known as aromatase. Figure from http://en.wikipedia.org/wiki/, enzymes have been added.*

One important enzyme in steroidogenesis is CYP19 (aromatase) which is responsible for the conversion of testosterone to estradiol and plays a key role in sexual brain differentiation. Another enzyme important for normal reproductive function is the reductive form of 17β-HSD (hydroxysteroid dehydrogenase) that converts

biologically weaker steroids into more active steroids, whilst the oxidative form of 17β-HSD changes active steroids into less active steroids. A variety of tissues express these enzymes, including liver, brain, adipose tissue, reproductive tract and breast tissue, so sexual differentiation and development is a extremely coordinated pattern (Whitehead and Rice, 2006).

Mechanisms of endocrine toxicity

Many POPs have the potential to interact with multiple targets in the endocrine system and their mechanisms of action include mimicking endogenous hormones (agonistic/antagonistic effects), altering hormone synthesis, transport, metabolism or secretion, and disrupt production or function of hormone receptors. Depending on the compound in action, more than one mechanism may be utilized by a single POP, i.e. a compound interfering with a steroid receptor activity, may also affect the activity of the receptor gene itself (Goksoyr, 2006).

POPs ability to mimic endogenous hormones may exert effect by binding to steroid receptors leading to agonistic and antagonistic effects. Estrogenic activity by binding to its receptor has been observed for PCB-138, -153 and -180, which can compete for ER and elicit oestrogenic activity (Bonefeld-Jorgensen et al., 2001). In addition, PCB-3, -6 and -17 and the corresponding hydroxylated metabolites (OH-PCB) have also been shown to bind ER in a recombinant yeast oestrogen assay. Estrogenic activity of PCBs was found to be 25- to 650-times less than for the corresponding OH-PCBs (Layton et al., 2002). *o,p'*-DDT is also a well known inducer of estrogenic activity by its binding to ER (Chen et al., 1997). Furthermore, some BFRs have also been investigated for estrogenicity *in vitro* in an oestrogen receptor-mediated reporter gene assay (ER-CALUX) which expresses luciferase upon cellular exposure to estrogenic compounds. It was found that BDE-30, -47, -51, -75, -100 and -119 were the most potent congeners for estrogenic activity, as well as a few OH-PBDEs (Meerts et al., 2001).

Coplanar PCBs and TCDD have also been associated with the oestrogen receptor. However, the mediated effect is antiestrogenic. Coplanar PCBs seem to bind directly to ER whereas TCDD mediates the effect through a cross-talk with the AhR by iDREs located in promoter regions of some oestrogen-responsive target genes (Safe, 2001;Hruska and Olson, 1989;Bonefeld-Jorgensen et al., 2001). Moreover, TCDD have also been shown to decrease ER density in rat (Hruska and Olson, 1989), and to disrupt human steroid production in granulosa cells by reducing estradiol secretion; which might be due to altered cell membrane permeability (Heimler et al., 1998). In the same aforementioned ER-CALUX assay, some PBDE congeners such as BDE-153, -166 and -190, were also found to be antiestrogenic by reducing E2-induced luciferase activity (Meerts et al., 2001).

Besides antiestrogenic effects of PCB-138, antiandrogenicity due to binding to AR have also been demonstrated (Bonefeld-Jorgensen et al., 2001). Furthermore, commercial mixtures of PCBs as well as PCB-31 and -42 have been shown to reduce androgen steroids *in vitro* by interfering with AR-mediated transcription (Portigal et al., 2002). Also, *p,p'*-DDE was found to act as a potential AR antagonist (Kelce et al., 1995).

Adversely altered levels of endogenous hormones by inducing or inhibiting enzymes important for synthesis or metabolism have also been demonstrated. *p,p'* –DDE has been found to stimulate aromatase activity in human granulosa cells (Younglai et al., 2004). DDT and *o-p*-DDE, as well as lindane, has also been shown to inhibit aromatase activity *in vitro* (Sanderson et al., 2002). In addition, environmentally relevant PCB mixtures have also been shown to reduce aromatase activity in rat brains (Hany et al., 1999). The mechanism of action for this inhibition is poorly studied, however, it is suggested that DDT and derivatives may inhibit the generation of cyclic adenosine monophosphate (cAMP), a second messenger for regulating aromatase expression and activity (Whitehead and Rice, 2006).

Critical windows of exposure

Exposure to relatively high doses of toxic compounds affecting the endocrine system may lead to marked disruption in adults. However, lower levels may also be harmful if exposure lasts over longer periods of time and/or at a critical time in development. The sensitivity of an individual to POPs interfering with the endocrine system is varying during different life stages. Induced effects in sensitive life stages of development may be hidden at birth and not revealed until later in life when the offspring reach sexual maturity or even in middle age.

The most vulnerable period to chemicals is not only depending on the period itself, but also on the type of chemical and its mechanism of action as well as on the species and organs responding to the endocrine signals. However, particularly foetus and infants appear to be more sensitive than adults, due to rapid growth and development. Several cases of impaired reproduction have been reported in fish and wildlife (Birnbaum, 1994;Colborn et al., 1993).

The formation of organs (organogenesis) during development is a highly vulnerable period and is regulated by endogenous steroid hormones together with other factors. The fact that many POPs have the ability to mimic endogenous hormones makes foetus very susceptible to these chemicals. In a goat study where low dose exposure with PCB-153 was used during gestation and lactation, endocrine disruption, including effects on puberty development, was found in female kids (Lyche et al., 2004b). Furthermore, *in utero* and gestational exposure to TCDD in male mice adversely affected prostate development (Lin et al., 2002). A maternal low level exposure *in utero* and during lactation has also been identified with a reduced growth and fertility of Oldfield mice (*Peromyscus polinotus*), as well as these effects were to be enlarged through multigenerational exposure (McCoy et al., 1995).

Transgenerational exposure effects

Embryonic development involves DNA-related mechanisms of inheritance such as methylation and chromatin remodelling, which is highly important for normal development. The majority of the germ cell DNA is methylated and demethylated in a specific pattern, while a small set of genes are imprinted and transmit a unique methylation pattern to subsequent generations via the male or female germline (Crews and McLachlan, 2006). Modification of methylation patterns during embryonic life may result in abnormalities and transgenerational disease state (Anway et al., 2005). Normally, hormones are known to epigenetically imprint genes in nonmammalian vertebrates (Crews and McLachlan, 2006). However, recently it was demonstrated that embryonic exposure to an endocrine disruptor (vinclozolin; pesticide) had the potential to cause a transgenerational effect on male fertility and testis function which lasted via three generations (Anway et al., 2005). The methylation patterns in four genes were different in the exposed animals compared to the controls. Additionally, embryonic exposure to an estrogenic compound resulted in an altered epigenetic imprinting in mice (Li et al., 1997).

2.4 AhR mediated toxicity

Animals and humans have developed a range of defensive mechanisms to toxic compounds found in the environment. Enzymes that contribute to the biotransformation and elimination of these compounds are induced in response to ligand-activated receptors that bind to DNA and influence gene expression for these enzymes. One kind of receptor, previously mentioned, the AhR, is activated by dioxin and dioxin-like compounds and is subjected to induce a variety of toxic and biological responses, including induction of biotransformation enzymes.

The Ah receptor was first identified in mouse liver and demonstrated specific binding to radiolabelled TCDD (Poland et al., 1976). Subsequent studies have shown that

AhR is associated to a number of other halogenated aromatic hydrocarbons that activate the AhR to different degrees (Poland and Knutson, 1982).

The signalling pathway of AhR mechanism is modelled in Fig. 2.8. The non-liganded inactive AhR remains in the cytosol as a complex associated to a dimer of Hsp90 (heat shock protein; chaperone), p23 (co-chaperone) and XAP2 (hepatitis B virus X-associated protein 2). The associated proteins are bound to a nuclear localization sequence (NLS) of the AhR preventing the non-liganded receptor to enter the cell nucleus (Fig. 2.8). When a ligand binds to AhR it undergoes a conformational change and the proteins dissociate, resulting in an open NLS and thus allowing translocation of liganded receptor into the nucleus. The nuclear AhR dimerizes with ARNT (AhR nuclear translocator protein) and this heterodimer acts as a functional transcription factor capable of interacting with regulatory sequences in DNA, known as dioxin or xenobiotic response elements (DREs; XREs), in promoters of CYP1A1 gene and other Ah-responsive genes (CYP1A2, CYP1B1, UDP-glucoronosyltransferase, glutathione transferase and aldehyde dehydrogenase). Additional recruitment of coactivators and general transcription factors leads to transcription and an altered gene expression of the subsequent genes resulting in a changed biological response (Casarett and Doull, 2001;Safe, 2001;Safe, 1994). In addition to the upregulation of biotransformation enzymes, dioxin-activated AhR has been found to cause a wide variety of toxic effects (Section 2.2 “Dioxins”).

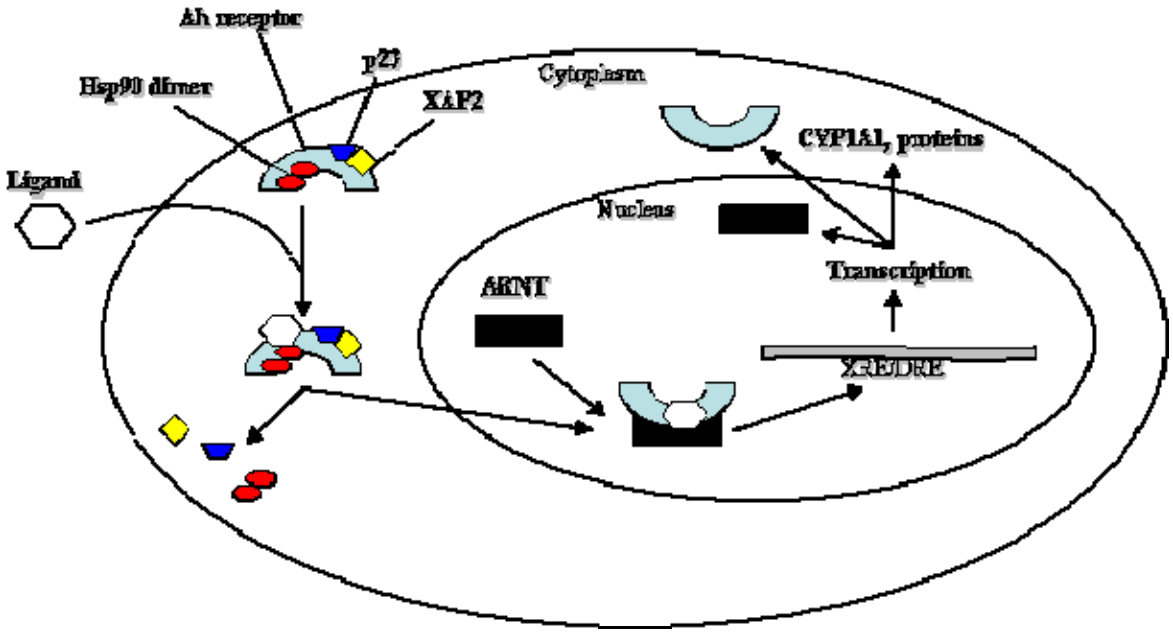


Figure 2.8: *Signalling pathway of AhR mediated gene expression. Upon ligand binding to the AhR-complex in the cytosol the associated proteins (Hsp90, p23, XAP2) separate and the liganded AhR enters the nucleus. After dimerization with ARNT, the AhR-ARNT heterodimer binds to xenobiotic or dioxin response elements activating transcription of the CYP1A1 gene and other biotransformation enzyme coded genes. This figure is modified from Mandal (2005).*

The AhR is present in several tissues in humans and other animals and therefore may exert toxic effects in many organs. Mediated effects of TCDD and dioxin-like compounds show strain and species differences as well as variations between tissue, cell, age, sex, and duration and windows of exposure (Mandal, 2005).

The expression of CYP1A1 is commonly used as a biomarker of dioxin exposure in fish, by measuring the enzymatic activity level as 7-ethoxyresorufin-*O*-deethylase (EROD) activity [Section 2.7; (Sarkar et al., 2006)].

2.5 The heavily polluted Lake Mjøsa

Reports on geographical levels of POPs in the environment shows enormous variations in the biota, where particularly aquatic species appear to contain among the highest detected levels (Law et al., 2006;Muir and Norstrom, 2000;Hale et al., 2003;Hale et al., 2001). A report by Norwegian Institute for Water Research (NIVA) from 2001 states alarmingly high concentrations of certain POPs, particularly PBDEs, in fish from Lake Mjøsa compared to the other observed lakes in Norway (Fjeld et al., 2001). Furthermore, high concentrations of PBDEs have been detected in humans that consumed fish from Mjøsa and a clear associations between the concentrations of PBDEs in serum and the intake of freshwater fish was found (Thomsen et al., 2006). The location of Lakes Mjøsa and Losna in Norway is presented in Fig. 2.9.

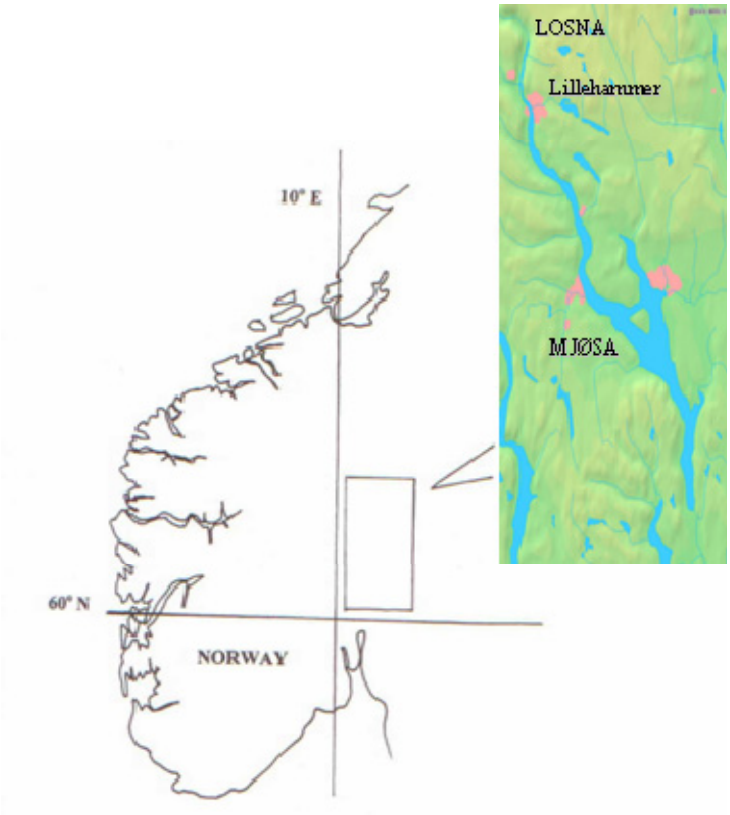


Figure 2.9: *Map over the southern part of Norway and the location of Lakes Mjøsa and Losna. Mjøsa is the largest lake in Norway and Lake Losna is a part of the Gudbrandsdaslågen and the major inlet to Lake Mjøsa near Lillehammer.*

In the aforementioned report by NIVA, levels of ΣPBDE for BDE-47 and -99 in burbot (*Lota lota*) liver from Lake Mjøsa were 1500-3900 ng/g lipid weight (lw) (Fjeld et al., 2001) in contrast to burbot livers from Lake Michigan, USA, that contains 2440 ng/g lw, which is also subjected to be a remarkably polluted area (Manchester-Neesvig et al., 2001). These levels were also relatively high compared to cod (*Gadus morhua*) livers from the Norwegian cost which contained 135 ng/g wet weight (ww) of the same two congeners. Another recent study by NIVA from 2004 demonstrated levels of $\Sigma PBDE_{10}$ in burbot livers from Mjøsa to be 21900 ng/g lw (Fjeld et al., 2004). This clearly shows the ongoing pollution which is most likely due to the industrial adjacent areas. In burbot livers from Lake Losna $\Sigma PBDE_{10}$ was detected to 49 ng/g lw, which reflects that Losna is regarded as a "clean" area compared to Lake Mjøsa.

Despite the decline in PCBs and DDT levels in the environment, these compounds are still found in a high concentration range in Lake Mjøsa. Reported by NIVA, trout (*Salmo trutta*) muscle tissue from Mjøsa was found to contain ΣPCB_7: 75 ng/g ww and liver burbot ranged between ΣPCB_7: 1300-1700 and 5800 ng/g ww depending on the capture location of the lake. In comparison, ΣPCB_7 levels in trout from other lakes in Norway, such as Randsfjorden, Ellasjøen and Øyangen, were 24, 715 and 24 ng/g ww, respectively, in the muscle tissue. In the same report, levels of ΣDDT correlated to the ΣPCB_7 levels in the same species as well as in the same areas (Fjeld et al., 2001). Again, trout and burbot from Lake Mjøsa contained the highest concentrations of ΣDDT, 61 and 1100-3700 ng/g ww respectively, compared to the other lakes where the trout muscle was found to contain 15.6 and 2.76 ng/g ww from Randsfjorden and Øyangen, respectively. The high DDT and PCB levels found in Lake Mjøsa suggest that there is an ongoing exposure in this area, most likely originating from agricultural and industrial sources.

Furthermore, ΣPCDD/PCDF and Σdioxin-like PCB_4 in burbot liver from Lake Mjøsa was found to be 313 and 4000 ng/kg ww, respectively in 2001 (Fjeld et al., 2001). In comparison, Σdioxin-like PCB_4 measured trout from Lake Michigan in 1998 was 141.5 ng/g ww (Hickey et al., 2006), which again demonstrates the pollution situation of Lake Mjøsa.

2.6 Mixture effects of POPs

During the past 100 years of science, toxicity assessment has focused mainly on characterization and regulation of individual chemicals. Although information about single-chemical effects is of high importance, contaminant like POPs does rarely appear as single congeners or as commercial mixtures in the environment. Hence, risk assessment for mixtures reflecting the environmental pollution is needed.

In general, the exposure of these compounds is below the levels causing toxicity. The single components that appear in complex mixtures may alter their mechanism of action once released into the environment. They may act independently or several compounds may interact to modulate the effects of the whole mixture. Toxicological interactions may result in either a weaker (antagonistic) or a stronger (synergistic) combined effect than would be expected from single compounds. A study with a binary mixture of xenoestrogens (EE2, a synthetic oestrogen and nonylphenol, a weak ER agonist) given to zebrafish, was found to act both additively and non-additively at environmental relevant concentrations (Lin and Janz, 2006). Complex mixture results are therefore more difficult to interpret.

To assess the toxicity effects of complex mixtures, it is important to have information of all components included in the mixture, as well as the mechanism of action for each compound. Therefore, testing of mixtures together with all individual components at one dose level may be helpful. Data on action or interaction of the individual components and impacts of these changes may sometimes be difficult to

interpret. Dose-response curves for each chemical is therefore important in order to do risk assessment of chemical mixtures.

2.7 Biomarker detection for pollution screening

Biomarkers have been developed in order to monitor aquatic pollution and study the impact of chemicals or toxins on marine and freshwater populations. A biomarker can be defined as measurements of body fluids, cells, or tissues that indicate presence of contaminants in biochemical or cellular term (Sarkar et al., 2006). Several biomarkers is available, however, this study focus on EROD and vitellogenin (Vtg) induction.

EROD

Many pollutants, such as dioxins and dioxin-like PCBs are potent inducers of xenobiotic-metabolizing enzymes including CYP1A (Jonsson et al., 2006). The induction of CYP1A is triggered via the AhR pathway and is measured in terms of EROD activity, in which EROD catalyzes the dealkylation of ethoxyresorufin to resorufin (Sarkar et al., 2006). In general, the expression of CYP1A is low, but greatly induced by AhR agonists in several tissues in different species. The liver has traditionally been used to detect EROD activity; however, methods for measuring EROD activity in gills have also been more common (Jonsson et al., 2006;Jonsson et al., 2002). The CYP1A gene has been sequenced and characterized in zebrafish and showed high identity with mammalian CYP1A (Yamazaki et al., 2002).

Vitellogenin

One method to assess exposure of oviparous species, such as fish, to oestrogens and oestrogenic compounds is to measure the yolk protein precursor Vtg (Sumpter and Jobling, 1995). During sexual maturation of the female fish E2 is synthesized in the gonads and is further transported to the liver where its binds to ER leading to

transcription of vitellogenin gene. The Vtg protein is transported to the oocytes, where the plasma Vtg levels normally indicate the maturation status of the female fish (Nilsen et al., 2004). It appears that male fish from environment has been found with elevated levels of Vtg, which in a normal situation express low levels of this protein. When male fish are exposed to oestrogens, it triggers the vitellogenin gene and increases the levels of Vtg; a highly responsive marker for oestrogens (Sumpter and Jobling, 1995). By measuring the Vtg levels in male fish it is possible to assess oestrogenic activity of pollutants. The zebrafish is an ideal test species for this purpose.

2.8 The zebrafish model for effect studies of POPs

Zebrafish (*Danio rerio*) is a tropical fresh water fish belonging to the minnow family (Cyprinidae) native to rivers in India and Pakistan. The zebrafish was chosen as the test species, due to a number of features making it attractive as a vertebrate model for toxicological reasons as well as for developmental biology and functional genomics studies.

The main benefits of using zebrafish in toxicology research is its small adult size (3-4 cm in length; Fig. 2.10), which greatly reduces husbandry space and costs. Furthermore, zebrafish are easily held in compact recirculation systems, breed continuously year-around and have a short generation interval (4-5 months). Each female can lay up to 300 eggs weekly, which are fertilized externally. Importantly, the embryos chorions are transparent (Fig. 2.10) allowing visualization of internal processes such as formation of organs inside the living organism. All life stages of the zebrafish have been well characterized from the embryo to adulthood in both sexes. They hatch within 2-3 days after fertilization and have a life span of 2 – 3 years (Alestrom et al., 2006;Kimmel, 1989). The zebrafish is a teleost fish closely related to Common carp (*Cyprinus carpio*), and is far closer to fish species of aquacultural interest, such as salmon, cod and trout, than any mammalian model organism (Dahm and Geisler, 2006). Research on growth, stress, and disease

resistance in the zebrafish can be expected to produce comparable results to aquacultural species.

Figure 2.10: *Photographs of zebrafish, from the left to the right; transparent embryo 24 hours after fertilization, hatched larva 72 hours after fertilization and an adult zebrafish approximately five months of age.*

Another major advantage is that the zebrafish genome is sequenced, though not completely finished or fully annotated, enabling its use in functional genomics research. The 7th assembly of the zebrafish genome predicts a genome size of 1.527.000.381 base pairs and contains over 31000 genes (The Danio rerio Sequencing Project, 2007). Microarray libraries based on genes or transcriptional units from the sequenced zebrafish genome are now commercially available, each including over 16000 zebrafish genes. For example, the Alestrom Zebrafish Lab offers slides printed with zebrafish 16k 65-mer oligo library to the community (The Norwegian Microarray Consortium, 2007). Genes important for normal reproduction and normal development, such as CYP19 and ER, together with genes responding to pollutants, like aromatic hydrocarbon response elements (AHREs) has been characterized and represented on the arrays.

The usefulness of zebrafish in toxicity testing has become extremely valuable. Responses to exposure of toxins can be measured at molecular, cellular, individual and population level, providing useful information for risk assessment. Furthermore,

the small size of the zebrafish makes it possible to do multi-generational exposure studies, which can give information about transgenerational effects.

2.9 Use of microarray in toxic effect studies

The microarray technology is an extremely useful tool in order to examine thousands of genes simultaneously at the expression level of mRNA. Basic principles and procedures of the microarray experiments are presented in Fig. 2.11. In cDNA and longoligo based microarrays, total RNA or mRNA is extracted and purified from the two samples to be compared and either green Cy3 or red Cy5 is incorporated during reverse transcription (Lee et al., 2005). The two labelled cDNA mixtures are combined in equal amounts and hybridized to the microarray, after which the slide is scanned. Intensity of each of spot is determined, where Cy3 and Cy5 signals are overlaid, yellow spots indicate equal intensity of the dyes. Moreover, the image analysis software computes the logarithm of the ratio of Cy3 and Cy5 intensity, where a positive log(Cy5/Cy3) ratio indicate upregulation of the Cy5-labelled transcript and a negative log(Cy5/Cy3) ratio indicate downregulation of the Cy5-labelled transcript. The images will further be used to identify differentially expressed genes using statistical software programs.

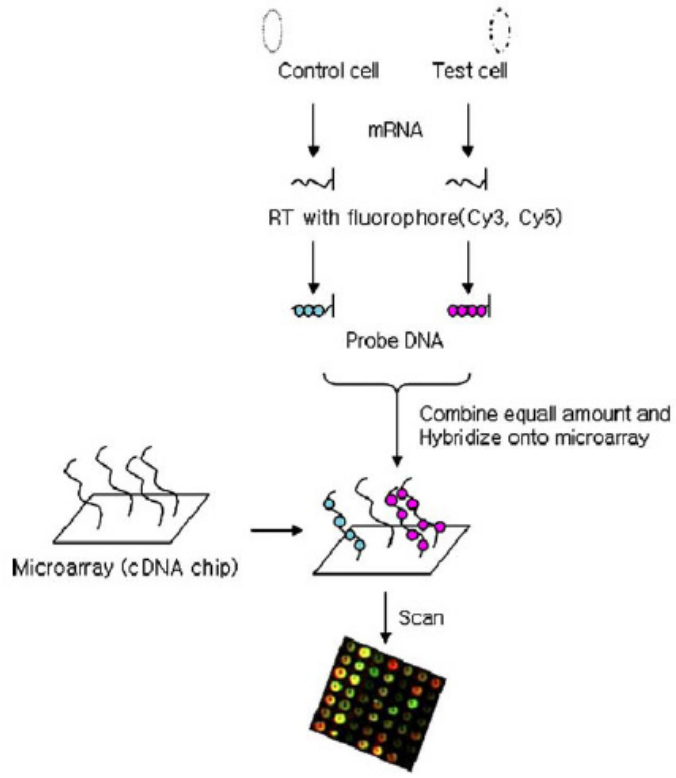


Figure 2.11: *Brief diagram of the procedure of cDNA microarray. mRNA is extracted and purified from the samples (i.e. control and treated) and dyed with their respective Cy3 or Cy5. The probe DNA is then combined in equal amounts and hybridized to the microarray, after which the chip is scanned and analysed for differentially expressed genes. Figure from Lee et al. (2005).*

It is essential that the microarray experiment is performed with cautiousness, especially when working with dual colour fluorescence where the endpoint is the intensity ratio between the two samples to be compared. However, care must also be taken in the experimental steps within the microarray set up where particularly the purity of the isolated RNA is one of the most critical issues for a successful microarray experiment. The use of herring sperm DNA ensures that the signal measured from each spot is precise to the particular probe sequence, and that background is minimized. Furthermore, spike control is also used for assessing the data quality (White and Salamonsen, 2005).

This technology is an extremely useful screening tool in order to assess environmental induced toxicity at the molecular level. Changes in gene expression patterns provide sensitive markers of toxicity and genes associated with disease or altered physiological condition can be identified. Furthermore, altered expression profiles during developmental stages are also essential when aiming to identifying critical windows of exposure.

Moreover, this technique is an important monitoring tool in order to detect presence of pollutants in aquatic environments and gain information of their mechanisms of action. Additionally, genes sensitive to toxic compounds may be identified as biomarkers when exposed to mixtures of pollutants. The microarray technology is more a useful tool for assessment of possible differences between diverse exposure groups in cells, tissues or organisms.

2.10 Aims of the study

The persistence and the increasing levels of POPs in the environment, together with documented adverse impact in fish, wildlife and humans, is a vast and complex problem. Many experimental studies have focused on individual compounds or commercial mixtures of POPs, which do not reflect the exposure situation in humans or wildlife. In the present study long-term exposure, low level exposure and exposure to real life mixtures of persistent organic pollutants harvested from aquatic environments were used to investigate exposure effects *in vivo*.

The main aim of this project was to study developmental and reproductive effects of extracts of brominated flame retardants and organochlorine chemicals harvested from burbot liver in Lake Mjøsa and Lake Losna.

Zebrafish was chosen as a model because aquatic products represent a major source of POPs intake for humans. Additionally, the zebrafish genome is sequenced and genomic methods, including gene expression profiling, are well established.

The specific aims were:

1. Establish a population of zebrafish suitable for exposure, where the model reflected exposure levels and routes occurring in the environment.

2. Identify as many compounds as possible in mixtures originating from contaminated fish and investigate bioaccumulation of POPs in the zebrafish.

3. Investigate demographic variables, such as survival, sex distribution, onset of puberty and length and weight of fish in relation to exposure.

4. Use the microarray technology in order to study tissue-specific gene expression profiles after long-term exposure.

5. Identify possible biomarkers, EROD and vitellogenin, related to the exposure scenario, as well as defining critical windows of exposure.

3. Materials and methods

3.1 Chemicals used for exposure

Mixtures of persistent organic pollutants (POPs) harvested from burbot (*Lota lota)* liver from Lake Mjøsa and Lake Losna (Fig. 2.9) was used for exposure of zebrafish during two generations.

Preparation of real life mixtures of POPs

Livers from burbots weighing 1-2 kg, caught in August 2004 in Lake Mjøsa and Lake Losna (Fig. 2.9; 61º 02’ N, 10º 27’ E and 61º 24’ N, 10º 13’ E, respectively), were transported on ice to the laboratory and stored at -20°C until processing at the Environmental Toxicology Laboratory at the Norwegian School of Veterinary Science. The laboratory is accredited for analysing the components reported here, according to the requirements of NS-EN ISO/IEC 17025:2000. Certified international reference materials (CRM 349 and 350, ICES cod liver oil and mackerel oil) are analysed regularly, with results within the given ranges. Spiked samples and the laboratory’s own seal (*Phoca groenlandica*) blubber reference sample are included in each series of analysis.

POPs were extracted according to the method described by (Brevik, 1978), in addition to the use of ultraturrax® after adding the solvents for optimising the extraction. Liver tissues were homogenized before adding of solvents, ultrasound extraction and centrifugation. Aliquots of the samples were used for lipid determination and clean-up with sulphuric acid (SA), and extracts from the burbot livers were analyzed for POPs (Appendix 1).

To prepare the Mjøsa and Losna stock solutions, livers were sliced with a scalpel and repeatedly frozen and thawed to room temperature, to separate the oil from the liver tissue, before centrifugation at 3 000 rounds per minute (rpm) for 10 min. 10 g of the clear oil was poured into a flask and diluted by adding 50 ml of cyclohexane (CHX) and 200 ml of concentrated SA before shaking repeatedly together with freezing and thawing, separating the cyclohexane from the acid and generating an organic phase containing the cyclohexane and lipids. The acid phase was removed and the volume reduced under N_2, and new acid added. This was repeated until the volume was reduced to 1 ml. In each step the CHX/SA batch was given at least two days for the SA to separate from the organic phase. The CHX was replaced by adding 10 ml of acetone and reducing the volume under N_2 to 1 ml. This was repeated four times and the final extractions were diluted to give the Losna treatment and the three different dose levels of the Mjøsa treatment. Aliquots of the Losna and Mjøsa High treatments were analyzed for POPs. Aliquots of the samples were used for lipid determination and clean-up with SA, and extracts from the burbot livers were analyzed for POPs (Appendix 1).

Chemical quantification of POPs

BDEs and HBCD were determined by gas chromatography-mass spectrometry (GC-MS) according to a method described by (Murvoll et al., 2005). PCBs, HCHs, HCB, chlordanes and DDTs were analysed using gas chromatography equipped with an electron capture detector (GC-ECD) according to a method described by (Andersen et al., 1999). The detection limit for BDEs was 2 ng/ml, and for HBCD 3 ng/ml. For PCBs it was from 0.4 - 4 ng/ml and for HCHs, chlordanes, HCB and DDTs it ranged between 0.5 and 3 ng/g.

The same method was later on used to analyse the brine shrimp *(Artemia salina)* and zebrafish after exposure.

The mean measured concentrations of POPs in burbot liver extracts from Lake Mjøsa (n = 20) and Lake Losna (n = 21) and in the stock solutions are given in Appendix 1. In the Mjøsa mixture the dominating pollutants were BDEs and HBCD, but also considerable amounts of PCBs and DDTs were found in burbot liver and in the resulting stock solution. The measured concentrations of toxins in burbot livers caught in Lake Losna were lower than in burbot livers from Mjøsa, except for HCHs and HCB, which were found at low concentrations in both locations. The extracted mixtures contained the same amounts of toxins although the levels were generally lower.

3.2 Experimental animals

Wild type zebrafish (*Danio rerio)* previously purchased from a local supplier, was used to generate a new (F0) generation, which subsequently was used to generate a second (F1) generation. The F0 and F1 generations were used in exposure experiments. The fish were kept in the Alestrøm zebrafish lab, Department of Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science.

Water conditions

Normal tap water was used to make reverse osmosis regulated water (RO-water) added 150 mg/l synthetic sea salt (Instant Ocean, Marineland, USA), 75 mg/l Sodium Bicarbonate (Sigma), 25 mg/l and Calcium Sulphate (Sigma). The pH was adjusted with 1 M Hydrogen Chloride (HCl) to 7.5, the conductivity was maintained at around 500 µS/cm and temperature set to 28ºC. Weekly ammonia (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) levels were measured and kept within the recommended ranges. Furthermore, the general hardness (gH) and carbonate hardness (kH) were monitored weekly and kept at levels around 4 and 5, respectively. The fish were maintained in systems providing recirculated water (Marine Biotech). The water was filtered through a particle filter, a charcoal filter and suspended bio filters. In addition, the

water was also irradiated by a UV-lamp. The water pressure in the system was set to ~6.5 GPM (Gallons per minute), equivalent to 6 water changes per hour. 10% of the system water was changed daily. A 13.5 hr: 10.5 hr of light: dark regime was maintained using an automatic timer.

Feeding regimé

Feeding was initiated when the larvae became free-swimming on 6 days post fertilization (dpf). The fish were fed four times daily. The first week of feeding, 6-12 dpf, fish were given fine powdered dry feed *ad libitum* (SDS100, Scanbur A/S, Nittedal, Norway). Between 13-18 dpf the fish were given brine shrimp *(Artemia salina)* in addition to SDS100, both twice daily. During the third week (19-25 dpf) SDS100 was given once a day and brine shrimp three times per day. Brine shrimp was given four times per day from 26 dpf and onwards.

Brine shrimp are species of aquatic crustaceans of the genus *Artemia,* which are commonly used as live food in aquariums due to high proteins content. They were obtained as dormant cysts, which are encased embryos that are metabolically inactive. When the cysts are placed into salt water they hatch within a certain time. The recommendations for hatching of the brine shrimp were followed (Scanbur A/S, Nittedal, Norway); a salt concentration of 28 ppt (parts per trillion, ~35 g/L), a brine shrimp concentration of max 10 g/L, a temperature of 28°C, a good oxygen supply and a hatching time of 24 hours. Five bottles (one for each group) with a water volume of 250 ml was prepared every morning. After 24 hours the brine shrimp was filtered, shell-rests discarded and new system water added. The brine shrimp given to the fish was administered via a plastic pipette and the same amount was given to every tank. As the fish grew, more brine shrimp was produced in accordance with recommendations from the manufacturer.

3.3 Experimental design

Establishment of zebrafish populations

Two males per female in 15 separate tanks were used in the breeding set up. The fish were transferred to marbled breeding tanks in the afternoon of the day prior to breeding, and spawning was triggered once the light was turned on the following morning. Eggs were collected and counted. Dead and unfertilized eggs were removed and viable eggs cultured in Petri dishes for two days, after which the eggs and the larvae were mixed and distributed into the exposure groups. These were labelled according to the treatment they were given in the F0 and F1 generations. At 6 dpf the numbers of larva in each tank was adjusted to 65 in the first (F0) generation and 80 in the F1 generation.

Initially there were 1625 zebrafish larvae in the F0 generation and 2800 larva in the F1 generation. The larvae were maintained in ¾ L tanks the first two weeks and then subsequently transferred to 1L, 2L and 6L tanks as they grew larger. All experimental groups in both generations consisted of five replicates (A-E).

Exposure through feed

Each week the fish were exposed to the POPs through their diet four times per day from Monday to Friday. On Saturday and Sunday the fish were given unexposed feed.

Pilot study

Prior to the exposure experiment a pilot study was carried out in order to improve information about the bioaccumulation of POPs in the brine shrimp and in the zebrafish. Adult zebrafish were divided in three groups and treated with respective doses of the mixtures (low and high of Mjøsa extract and one of Losna extract) through their diet for 31 days. Sampling of the fish was conducted at day 10, 20 and

31 of the exposure and later on analysed for BDE-47 and PCB-153 (Appendix 2). The results from the pilot study provided basis for determining the levels of Mjøsa and Losna mixtures that would give environmental relevant concentrations in the zebrafish after long term exposure.

Exposure of baby powder (SDS100)

Five batches of SDS100 fodder was prepared; labelled Mjøsa High, Mjøsa Medium, Mjøsa Low, Losna and Control, for the first three weeks of feeding. To ensure the baby powder was free of toxins, it was analysed and found it contained less than 1 ng/g wet weight of all components. The fat content of the feed was 10%. Mixtures were added abundantly to obtain the same concentration as in the burbot liver oil, which gave the Mjøsa High and Losna feed. 1/8 and 1/32 amounts of the Mjøsa High gave Mjøsa Medium and Mjøsa Low respectively. The fifth group (Control) was only treated with the solvent (CHX). Plentiful amounts of the CHX and mixtures were added to the batches of baby powder to ensure everything was soaked. Subsequently, the solvent was evaporated under a gentle stream of N_2 until the feed was completely dried. In the same manner the baby powders for the F1 generation were prepared and labelled Mjøsa High, Losna and Control.

Exposure of brine shrimp (*Artemia salina*)

A total of five 250 ml bottles with brine shrimp were prepared every morning containing 250 µl mixtures or solvent, and then labelled accordingly with Mjøsa High, Mjøsa Medium, Mjøsa Low, Losna and Control. The brine shrimp were being exposed for 24 hours during hatching. From the pilot study the observed bioaccumulation of BDE-47 and PCB-153 in the zebrafish indicated that the highest Mjøsa mixture after administered for several months could be even higher for achieving the same levels as found in the environment. Mixtures were dissolved in acetone and a maximum concentration of the solvent in the brine shrimp bottles was 0.1% (250 µl acetone/250 ml water). The Mjøsa High stock was adjusted through evaporation, and calculated to contain 4000 ng/0.250 ml, which gave a concentration of 16 000 ng/ml of BDE-47. The Losna treatment was prepared in the same manner

as the Mjøsa High extract, while 1/8 and 1/32 amounts of Mjøsa High gave Mjøsa Medium and Mjøsa Low, respectively. As the fish grew, more brine shrimp was produced in accordance with recommendations from the manufacturer. Exposure of the brine shrimp for the F1 generation was performed in the same way, and labelled Mjøsa High, Losna and Control.

The zebrafish model

The F0 generation included five experimental groups: Mjøsa High, Mjøsa Medium, Mjøsa Low, Losna and Control, respectively (Fig. 3.1), indicating that the mixture originating from Mjøsa burbot was used in three exposure dose levels and that the Losna mixture was used only in a single dose.

The exposure dose levels of the Mjøsa mixture (Mjøsa High, Mjøsa Medium and Mjøsa Low, respectively) aimed at achieving a wide range of zebrafish tissue concentrations of POPs, including concentrations higher than (Mjøsa High), equal to (Mjøsa Medium) and lower than (Mjøsa Low) environmentally relevant concentrations. The exposure dose level of the Losna mixture was intended to serve as a natural control reflecting POP levels present in fish from a relatively clean environment (Lake Losna). One group of fish given feed only exposed to the solvent (acetone) of POPs served as a control.

The second (F1) generation was generated from three exposure groups of the F0 generation: Mjøsa High, Losna and Control. Based on the exposures in the parent and offspring (F0 - F1) generations' seven exposure groups were generated: Control-Control, Losna-Control, Mjøsa High-Control, Control-Losna, Losna-Losna, Control-Mjøsa High and Mjøsa High-Mjøsa High (Fig 3.1), the first name indicating parental (F0) exposure and the second indicating exposure of the offspring (Fig. 3.1).

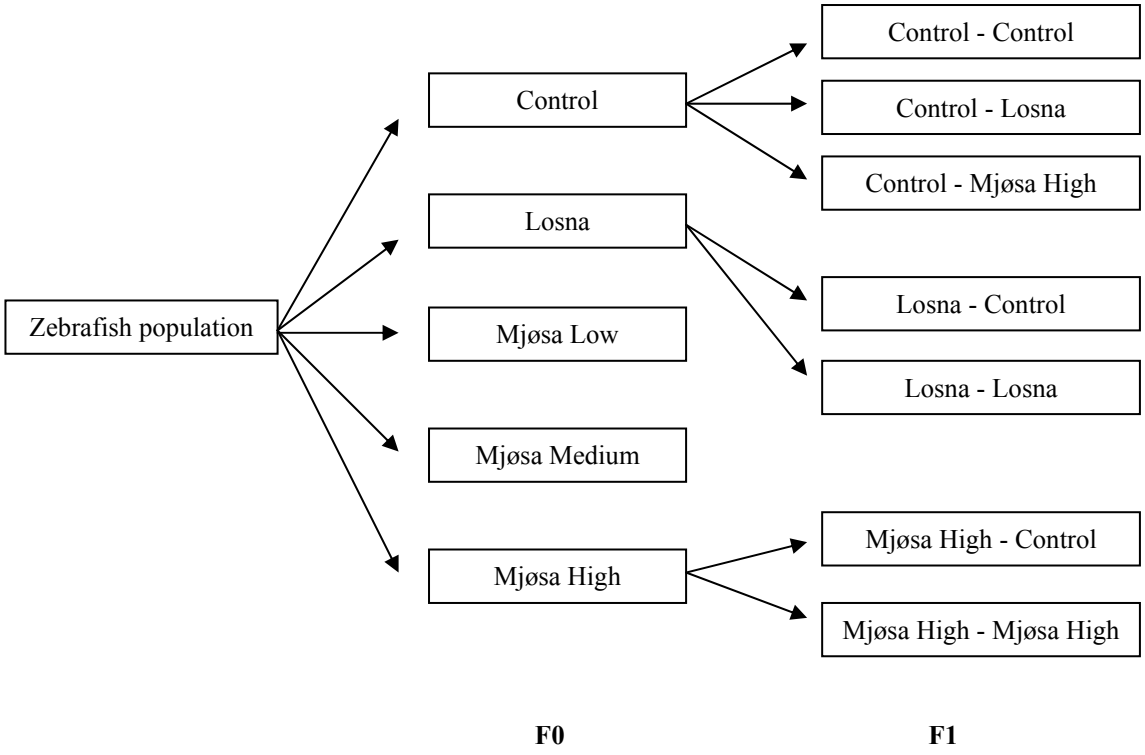


Figure 3.1: *An overview of the exposure experiment for two generations. The zebrafish population was divided into five groups in the first generation (F0), while only Control, Losna and Mjøsa High groups gave rise to the second generation (F1).*

Exposure of the fish was carried out from the day they started to eat (6 dpf) until they reached sexual maturity (Fig. 3.2). Daily records of survival of the fish were performed. After approximately four months of age the fish were tested for sexual maturity by placing boxes containing marbles inside the tanks, and everyday the boxes were checked for eggs. When fish from all tanks had produced eggs it was assumed that most of the fish were sexually mature. In all cases, sexual maturity of all fish was achieved after 5 months. At that point, harvesting of fish tissues was commenced.

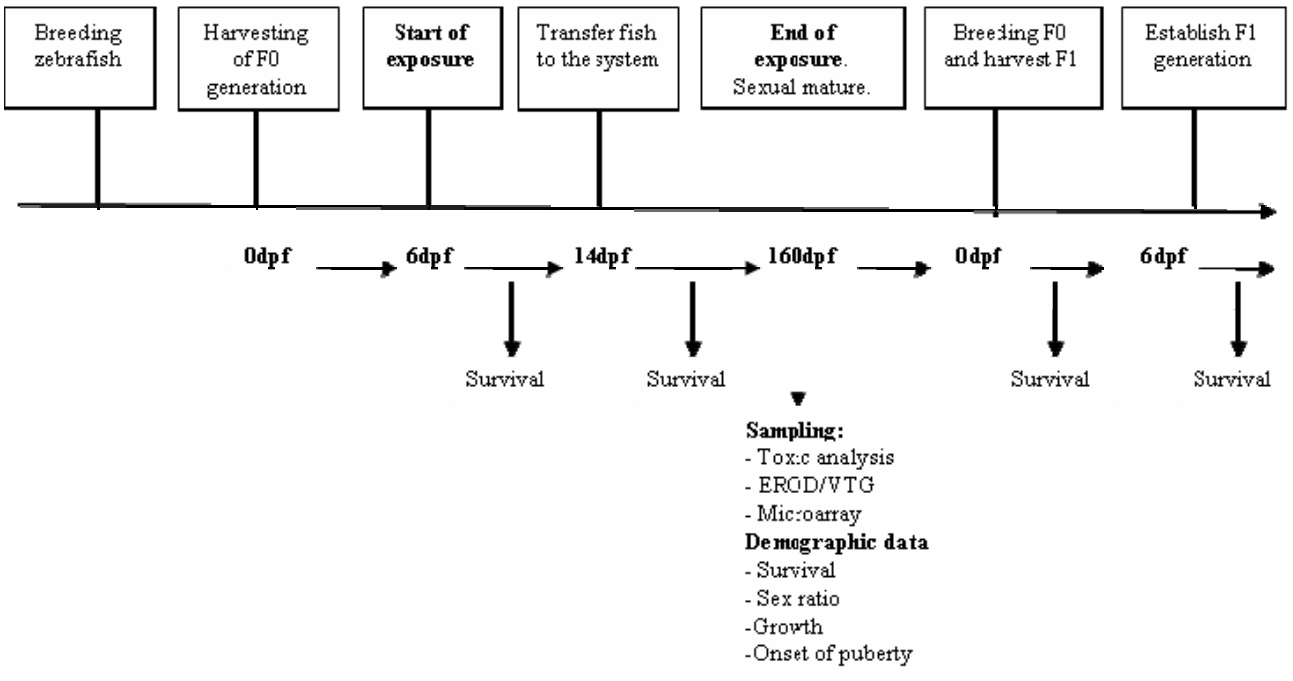


Figure 3.2: *Flow diagram of exposure experiment. Sampling was conducted when the fish became sexual mature at 160 dpf. The survival was monitored daily.*

3.4 Chemical analysis

One female and one male fish from each replicate were euthanized with an overdose of tricain methanesulfonate (MS 222, Sigma) and analysed for toxin exposure together with control and exposed artemia. BDE, HBCD, PCB, HCH, HCB, chlordane and DDT levels were determined as described in Section 3.1.

3.5 Studied endpoints

All fish sampled for studies of different endpoints were euthanized with an overdose of tricain methanesulfonate (MS 222, Sigma). Harvested fish materials were used for measuring chemical components, gene expression analysis, and detection of biomarkers. Additionally, demographic variables were recorded.

Demographic endpoints

Measurements of various demographic endpoints were performed in order to look at effects on whole-organism responses. The results obtained from Control-Mjøsa High exposed fish in the F1 generation excludes replicate D and E due to an undiscovered higher density of fish in the mentioned tanks, which significantly affected the mortality rates (data not shown).

Survival

The survival of the fish was monitored daily until sampling was initiated at 163 and 153 dpf for the F0 and F1 generation, respectively. Records were maintained for the numbers of dead or removed larvae/fish.

Sex distribution

Before sampling was initiated the genders of all fish were phenotypically determined. Males are torpedo shaped and have golden stripes in between the blue stripes, while females have silver coloured stripes and a whitish, bigger belly. In addition, mature females have a well-developed uro-genital papilla.

Onset of puberty

Each replicate (tank) was checked for spawning weekly from week 15-23 after fertilization. Onset of puberty was defined when spawning was observed for the first time in each exposure group.

Total body mass and length of harvested fish

In order to evaluate the effects of the mixtures on fish growth, total body mass and length were measured for all fish that had been sampled for different analyses.

Gene expression analysis

Micro dissection

Six male fish from each replicate were used to carry out gene expression analysis. Testes, liver and brain from each fish were dissected out for tissue-specific measurements. The design of the microarray analysis is given in Fig. 3.3. Five organs originating from one fish per replicate were pooled together and constituted one sample for the microarray analysis. Totally there were six testis samples and six liver samples from each group. Brains did not contain enough total RNA for microarray analysis. The dissection was performed on ice, and all the samples were kept on liquid nitrogen until storage at – 80ºC. Microarray results were only obtained from Control, Mjøsa High and Losna groups.

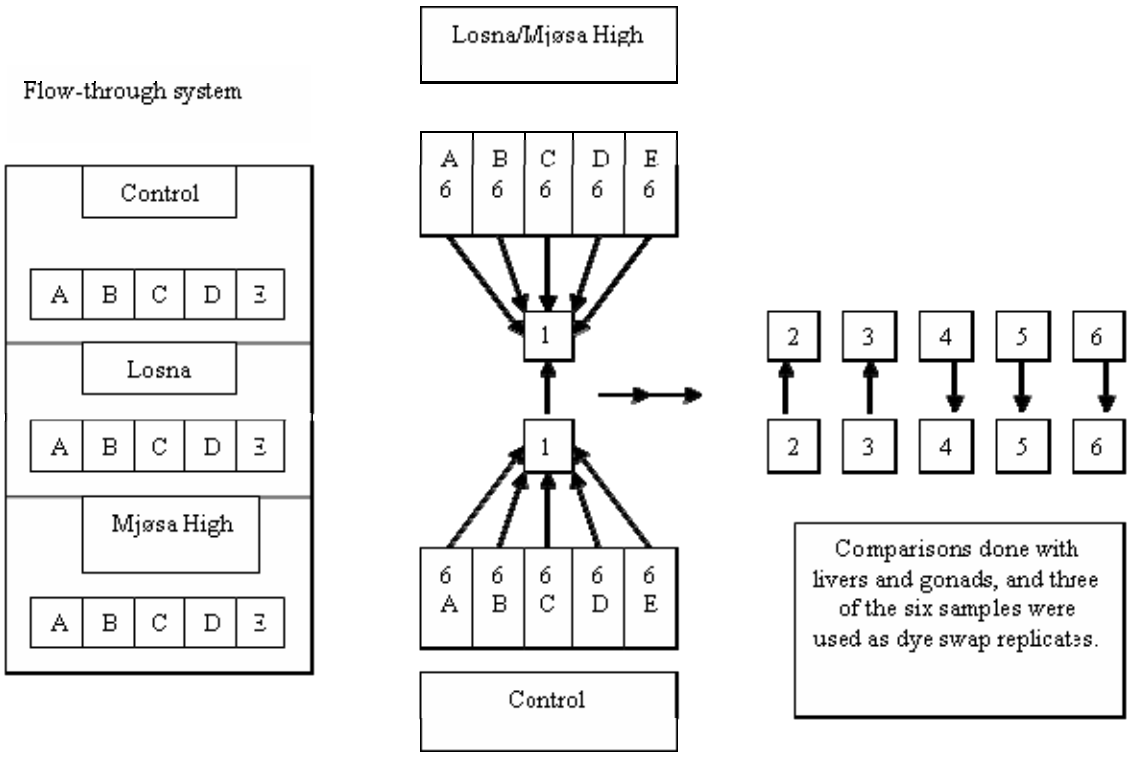


Figure 3.3: *Design of the microarray experiments. Testis and liver from one fish per replicate was pooled (pool 1-6). In three of the pooled samples, mRNA from controls treated fish were labelled with Cy3 and Cy5 respectively, while in the other three pooled samples, mRNA from controls and treated fish were labelled with Cy5 and Cy3 respectively, making dye switch replicates.*

RNA isolation

Extraction with Trizol

Total RNA was extracted from the organs using Trizol (Invitrogen). Before the samples were thawed, 1 ml of trizol was added to the frozen tissues (sample:trizol 1:10), and transferred to new microtubes containing magnalizer beads. The samples were homogenized two times for 50 seconds at frequency of 25 using Roller Mixer SRT1, United Kingdom. After 5 min incubation at room temperature, 200 µl chloroform was added to each tube and mixed thoroughly. In order to separate the aqueous phase (containing RNA) from the organic phase, the samples were centrifuged at 4000 rpm for 15 min at 2-8ºC. The aqueous phase of each sample was transferred to a fresh microtube and then an equivalent volume of isopropanol (approximately 400 µl) was added. The tubes were mixed prior to 10 min incubation at 15-30ºC. The samples were then centrifuged at 4000 rpm for 10 min at 2-8ºC. Supernatants were removed and pellets were washed in 75% ethanol. The tubes were vortex for a few seconds to loosen the pellets. Centrifugation was conducted at 4000 rpm for 5 min at 2-8ºC followed by removal of the supernatants. Pellets were allowed to air dry for 5 min. in the hood. After which 50 µl of RNAse free water (Qiagen) was added to the tubes and subsequently RNA concentrations were measured (Section "Nucleic acid measurements").

Before proceeding to the next step, each sample was DNase treated (RNase-Free DNase Set, Qiagen) for 20 min at room temperature.

Purification with RNeasy Kit

After isolation, the total RNA was further purified with RNeasy Kit (Qiagen). The manufacturers' protocol was followed ["RNA Cleanup"; (QIAGEN, 2007)] and the samples were eluted with 50 µl of RNAse free water (supplied with the kit). RNA concentrations and quality were measured using the NanoDrop. Afterwards the samples were aliquot into three tubes, one with 2 µl for RNA quality validation (Section "RNA quality check") and two aliquots with the same volume (2 x 24 µl) for microarrays analysis. The samples were stored at -80ºC until required.

Nucleic acid measurements

To measure total RNA concentration the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, USA) was used. Only 1 µl of the final elution of the purified RNA was added to the instrument and the optical density (OD) for nucleic acids was measured at 260 nm. The software calculates nucleic acid concentrations (ng/µl) and 260/280 nm and qualitative 260/230 nm ratios. These ratios were used as indicators for the presence of contamination in the samples. The 260/280 nm ratio should be around 1.8 and 2.0 for DNA and RNA, respectively, while the 260/230 nm ratio should be between 1.8 and 2.2, respectively.

RNA quality check

The "Agilent 2100 Bioanalyzer" (Agilent Technologies, California, USA) was used to examine the quality of purified RNA samples. Due to the omnipresence of RNases, and the instability of RNA, integrity and degradation checks are essential steps before RNA dependent applications. Briefly, the 2100 bioanalyzer is a capillary based gel electrophoresis system, capable of qualitative and quantitative analysis of RNA. The 2100 bioanalyzer expert software generated the RNA Integrity Number (RIN), ratios of ribosomal RNAs (28s/18s), gel-like images, electropherogram and tabular formats, which were used in the assessment of RNA integrity.

RNA 6000 Assay Guide

"RNA 6000 Nano LabChip®Kit" was used and the assay guide followed according to manufacturers instructions (Fig. 3.4).

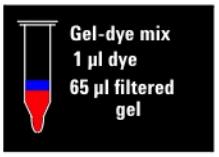
Agilent RNA 6000 Nano Assay Protocol - Edition August 2006

Preparing the Gel

1 Pipette 550 µl of RNA 6000 Nano gel matrix (red ●) into a spin filter.
2 Centrifuge at 1500 g ± 20 % for 10 minutes at room temperature.
3 Aliquot 65 µl filtered gel into 0.5 ml RNase-free microfuge tubes. Use filtered gel within 4 weeks.

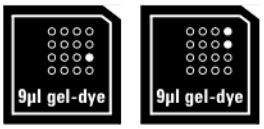
Preparing the Gel-Dye Mix

1 Allow the RNA 6000 Nano dye concentrate (blue ●) to equilibrate to room temperature for 30 min.
2 Vortex RNA 6000 Nano dye concentrate (blue ●) for 10 seconds, spin down and add 1 µl of dye into a 65 µl aliquot of filtered gel.
3 Vortex solution well. Spin tube at 13000 g for 10 min at room temperature.



Loading the Gel-Dye Mix

1 Put a new RNA 6000 Nano chip on the chip priming station.
2 Pipette 9.0 µl of gel-dye mix in the well marked G.
3 Close chip priming station.
4 Press plunger until it is held by the clip.
5 Wait for exactly 30 seconds then release clip.
6 Pipette 9.0 µl of gel-dye mix in the wells marked G.
7 Discard the remaining gel-dye mix.

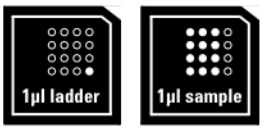


Loading the Agilent RNA 6000 Nano Marker

1 Pipette 5 µl of RNA 6000 Nano marker (green ●) in all 12 sample wells and in the well marked ◈.

Loading the Ladder and Samples

1 Pipette 1 µl of prepared ladder in well marked ◈.
2 Pipette 1 µl of sample in each of the 12 sample wells. Pipette 1 µl of RNA 6000 Nano Marker (green ●) in each unused sample well.
3 Put the chip horizontally in the adapter and vortex for 1 min at 2400 rpm.
4 Run the chip in the Agilent 2100 bioanalyzer within 5 min.



Technical Support IIn the U.S./Canada: 1-800-227-9770 (toll free); Isca-ibs-support@agilent.com. In Europe: call your local Customer Care Center; bio_solutions@agilent.com. In Japan: 0120 477 111; yan_ccr@agilent.com. In Asia Pacific: call your local Customer Care Center; Bioanalyzer_ap@agilent.com

Further Information Visit Agilent Technologies' unique Lab-on-a-Chip web site. It is offering useful information, support and current developments about the products and the technology: http://www.agilent.com/chem/labonachip.

Figure 3.4: *RNA 6000 Nano Assay Protocol Quick Reference Guide (Agilent Technologies) for validation of the RNA samples. Figure from http://www.agilent.com/chem/labonachip.*

RNA amplification

Amplification of RNA was performed using "Amino Allyl MessageAmp™ II aRNA Amplification Kit" with Cy™3 and Cy™5 dyes (Ambion). The manufacturer's protocol was followed. An overview of the procedure is presented in Fig. 3.5.

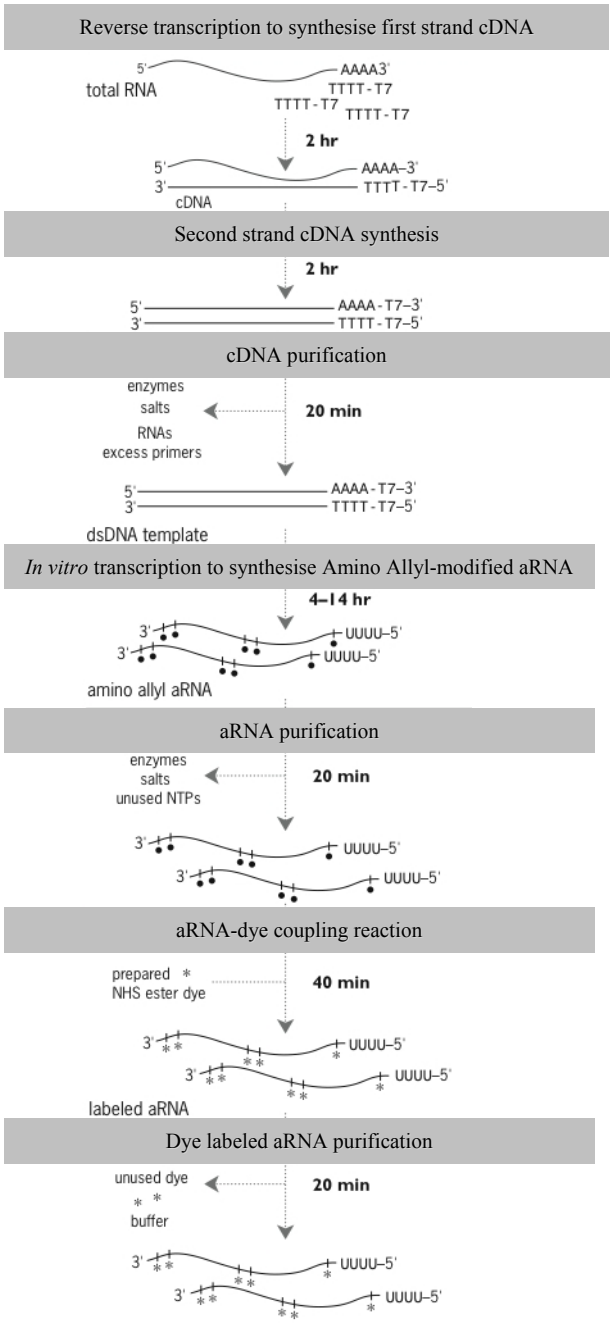


Figure 3.5: *Overview of the Amino Allyl MessageAmp II aRNA Amplification Procedure. First, the RNA is reverse transcribed to synthesise first strand cDNA, after which second strand cDNA is synthesised. cDNA is purified before in vitro transcription to produce Amino Allyl-modified aRNA where aaUTP is incorporated into aRNA. After purification of aRNA, aRNA-Dye coupling takes place between the aminoallyl-modified UTP residues on the aRNA and Cy3 and Cy5 dyes. Dye labelled aRNA is purified. Figure is modified from http://www.ambion.com.*

First Strand cDNA Synthesis

The total RNA was reversed transcribed using a T7 Oligo (dT) primer. To synthesise first strand cDNA by reverse transcription it was used 1 µg (at least 500 ng) of total RNA (max 10 µl). 1 µl spike controls and 1 µl of T7 Oligo (dT) primer were added to the sample tubes. If necessary, DEPC was added to a final volume of 12 µl. The samples were then incubated for 10 min at 70ºC in a thermo cycler with lid on. During the incubation a master mix (with 2-5% overage for pipetting errors) was prepared (Table 3.1).

Table 3.1: *The master mix for synthesis of first strand cDNA*

Component	Amount/reaction µl
10x First Strand Buffer	2
Ribonuclease Inhibitor	1
dNTP mix	4
Array Script	1
Total	8

To each RNA sample, 8 µl of the master mix was added and mixed by pipetting. Then they were incubated in a thermo cycler with lid on, but not clamped down, at 42ºC for 2 hr. After incubation the samples were briefly centrifuged and placed on ice.

Second Strand Synthesis

For the second strand synthesis the following master mix was prepared (with 2-5% overage for pipetting errors; Table 3.2).

Table 3.2: *The master mix for synthesis of second strand cDNA*

Component	Amount/reaction µl
Nuclease free water	63
10x Second Strand Buffer	10
dNTP mix	4
DNA polymerase	2
Rnase H	1
Total	80

80 µl of the master mix was added to each tube. The samples was mixed and placed in a thermo cycler at 16ºC for 2 hr with lid completely off.

Double Stranded cDNA Cleanup

After 2 hours incubation the double stranded cDNA was purified. 3-5 ml of nuclease free water was preheated to 50-55ºC for at least 10 min prior to use. 100 µl of the second strand reaction was transferred into microtubes, and kept on ice. 250 µl of cDNA Binding Buffer was added to each cDNA sample. The samples were then transferred to the centre of cDNA filter cartridges and centrifuged for 1 min at 10000 g. The flow-through was discarded and the tube was re-used. 500 µl of cDNA Wash Buffer (supplemented with ethanol) was added to the cartridge and centrifuged for 1 min at 10000 g. Again, the flow-through was discarded and the samples were centrifuged for 1 min at 10000 g in order to remove trace amounts of ethanol. Filter cartridges were placed in cDNA Elution tubes, 18 µl of preheated nuclease free water was added on top of each filter and incubated at room temperature for 2 min. The purified cDNA was eluted by centrifugation at 10000 g for 1 min.

In Vitro Transcription

The resulting cDNA was immediately used for *in vitro* transcription in the presence of Cy3- or Cy5-UTP, which were incorporated into aRNA. The samples were kept on ice while preparing the master mix (Table 3.3).

Table 3.3: *The master mix for in vitro transcription of aRNA*

Component	Amount/reaction µl
ATP,CTP,GTP mix (25 mM)	12
Aminoallyl-UTP (50 mM)	3
UTP solution (50 mM)	3
T7 10x RT buffer	4
T7 enzyme mix	4
Total	26

26 µl of the master mix was added to each tube. The tubes were mixed by flicking and centrifuged briefly. Incubation at 37ºC in a hybridization oven was performed for

14-16 hr. An internal thermometer was used to be sure the temperature was correct. The reaction was stopped by adding 60 µl nuclease free water to each RNA sample, making a final volume of 100 µl.

aRNA Purification

The amplified and labelled RNA (aRNA) was purified. Approx. 3 ml of nuclease free water was preheated at 50-60ºC for at least 10 min. 350 µl aRNA Binding Buffer followed by 250 µl 95% ethanol was added to each sample, and mixed by pipetting. The entire sample was the applied to the equilibrated aRNA filter cartridge and centrifuged for 1 min at 10000 g. The flow-through was discarded and tube was reused. 650 µl of aRNA Wash Buffer was added to each filter and centrifuged for 1 min at 10000 g. Again, the flow-through was discarded; the tube was reused and centrifuged for 1 min at 10000 g. The cartridge was then transferred to clean aRNA Collection Tube and the aRNA was eluted by adding 100 µl of preheated water to the centre of filter. After incubation at room temperature for 2 min the samples were centrifuged for 1.5 min at 10000 g. Lastly, the amplification yield of the aRNA and incorporation efficiency were measured using NanoDrop ND-1000 spectrophotometer, and samples placed at -80ºC.

Microarray analysis

Gene expression was analysed for the pooled samples from the control group versus respective pooled samples from Losna and Mjøsa High treated fish (design shown in Fig. 3.3) using arrays printed by The Norwegian Microarray Consortium. Oligonucleotides obtained from the Zebrafish Oligo Library (Cat#XEBLIB384, Sigma Genosys) were printed on glass slides. The library comprised 16 399 65-mer oligos [16 288 LEADS clusters (genes) and 171 controls]. The LEADS software platform clusters and assembles known mRNAs transcripts.

For the microarray analysis, Ambition MessageAmpII Amino Allyl with Cy Dyes and Amersham CyDye Post-Labelling Reactive Dye Packs were used. Manufactures instructions were followed.

Amino Allyl Coupling

Dual colour coupling to exposed samples and control sample was repeated by swapping the dyes. Three samples got one colour and three got the other colour.

11 µl of high grade DMSO were added to one tube of Cy3 or Cy5 dye supplied with the kit, followed by thoroughly vortexing. The dye was kept in the dark until use (preparation of dye should not take place >1 hr before use) and it was important to ensure that no water contaminated the Cy dye/DMSO mix at any point. 5-20 µg of amino allyl aRNA were added to microtubes and Speedvaced until completely dry. Once dry, it was removed from the Speedvac and kept on ice. To the dried RNA, 9 µl of couplings buffer was added, and the tubes was transferred to a 42ºC water bath and mixed thoroughly at regular intervals to resuspend the aRNA. 11 µl of the prepared Cy dye/DMSO was added to each sample and incubated in a covered box at room temperature for 1 hr. To quench the reaction 4.5 µl Hydroxylamine was added to each tube and incubated for 15 min in the dark. 5.5 µl of nuclease free water was added to the samples making a final volume of 30 µl.

Labelled aRNA Purification

Nuclease free water was preheated to 50-60ºC for a minimum of 10 min prior to use. 105 µl of aRNA Binding Buffer followed by 75 µl of ACS grade 95% ethanol were added to each tube. Samples were mixed gently and applied to equilibrated labelled aRNA filter cartridges before centrifugation for 1 min at 10000 g. The flow-through was discarded and 500 µl of aRNA Wash Buffer was added to each filter. The tubes were centrifuged for 1 min at 10000 g. Again, the flow-through was discarded and centrifuged for 1 min at 10000 g to remove trace amounts of ethanol. The cartridges were transferred to fresh labelled aRNA Elution Tubes and 70 µl of the preheated water was added to the centre of the columns. After incubation for 2 min at room temperature the tubes were centrifuged for 1.5 min at 10000 g to elute the purified aRNA.

Analysis of fluorescence-labelled cDNA

To measure the amount of cDNA produced (ng/µl) and dye incorporated (pmol/µl of Cy3 and Cy5), the NanoDrop ND-1000 spectrophotometer was used. The optical density of purified probes can be measured at 260 nm for nucleic acids, 550 nm for Cy3 and 650 nm for Cy5, and the NanoDrop software automatically calculates the concentrations of cDNA. Based on these measurements, the amounts of required cDNA for hybridization were determined.

Hybridization

Slides were incubated in 0.2 µm-filtered pre-hybridization buffer (Appendix 3) at 42°C for 30-60 min. Prior to use, they were washed at room temperature twice with nuclease-free water and once with isopropanol. Slides were quickly transferred to empty falcon tubes and centrifuged dry at 1 000 rpm for 1 min.

The hybridization volume was set to 450 µl and maximum volume of the samples was 225 µl. The corresponding Cy3 and Cy5 labelled samples were mixed together and nuclease free water was added to make the volume 216 µl. 9 µl of 25x Fragmentation Buffer (Agilent) was added to samples before incubation for 30 min at 60ºC. For each slide, 4.5 µl sonicated herring sperm DNA (10 µg/µl, Promega) was added to 220.5 µl of 2x hybridization buffer (Agilent), giving a final concentration of 0.1 µg/µl. 225 µl of sample was combined with 225 µl the prepared 2x hybridization buffer, making a total volume of 450 µl. After centrifuged at 13000 rpm for 1 min, the samples were transferred to cover slides placed in the microarray hybridization chambers. The printed slides were put on top, chambers were tightened and the arrays were inspected and gently agitated to remove immobile air bobbles before placing them in a rotation oven (3 rpm) at 60ºC for 16-20 hours.

Washing

After hybridization, washing was performed to remove unhybridised labelled cDNA molecules from the slide surface in order to reduce background. Three 50 ml falcon tubes per slide were filled with 50 ml of 0.2 µm-filtered Wash solution 1 (Appendix 3) and two 50 ml falcon tubes per slide were filled with 50 ml of 0.2 µm-filtered

Wash solution 2 (Appendix 3). Hybridization chambers were quickly dismantled and both slides were transferred to falcon tubes containing Wash solution 1. The cover slides became released and removed from the printed slides. Slides were then transferred to the second falcon tubes with Wash solution 1, wrapped in aluminium foil (preventing photo bleaching) and placed on a rotation machine (Roller Mixer SRT1, United Kingdom) with slow rotation. After 5 min incubation, the slides was transferred to new tubes with Wash solution 1 and incubated with rotation for an additional 5 min. The slides were washed twice in the same manner with Wash solution 2. After the last wash, the slides were transferred to empty falcon tubes and centrifuged dry at 1000 rpm for 1 min.

Analysis of microarray results

Scanning

The slides were scanned with a GenePix 4000B scanner (Molecular Devises, California, USA) and images were processed using the GenePix Pro software. Spot fluorescent intensities was measured at wavelengths 532 nm (Cy3) and 635 nm (Cy5) and converted to an electric signal, which were displayed as images. The intensity of arrays were adjusted to minimize the background, maximize the intensity of spots and to avoid saturated (white) spots. The Cy5/Cy3 ratio (Cy5 = red dye; Cy3 = green dye) for each spot was calculated by the software. The software automatically assigned gene identities to corresponding spots and associated data.

Normalization

The raw data generated by the GenePix software was imported into the *LimmaGUI* package incorporated in the statistical software R. *LimmaGUI* (Linear Models for Microarray Analysis) is a Graphical User Interface (GUI) based on linear modelling of data from two-colour spotted microarray experiments and provides data analysis and normalization for cDNA microarray data and analysis of differential expression. It log transforms the raw intensities of the spots in order to give a more symmetrical distribution about the mean values of red (R) and green (G) intensities, the log differential expression ratio is expressed as $M = \log_2(R/G)$ and the log intensity of

each spot as $A = 1/2\log_2(RG)$. Dye-swaps was taken into account, which means that one of the M values had to be multiplied by -1 before taking the average.

To perform the analysis *LimmaGUI* require a GAL (GenePix Array List) file, a RNA Target file and a Spot Type file; all must be put in as tab-delimited text files. Information about the files is given in Table 3.4.

Table 3.4: *The files required for the analysis of microarray data in limmaGUI.*

File type	Content
GAL file	Name and identify each spot on the array
RNA Target file	List the microarray hybridizations and contain slide number, file name (giving the output file from the image-analysis), Cy3 and Cy5 dyed samples (control or treated with respective colour) and date of scanning
Spot Type file	Optional, colour-code genes and controls

Normalization was carried out to adjust microarray data for effects which arise from variations in the technology rather than biological differences, i.e. imbalance of dyes labelling, non-uniform hybridization and printing variations. Therefore, normalization within an array by using Print-tip Loess method as well as between arrays by using a scaling of log-ratios was performed. The Print-tip Loess method compute a local regression to the relation between M and A values for each array and normalize each M-value by subtracting it from the loess value, so that both spatial effects and dye biases are removed. The scale normalization between arrays gives each array the same median-absolute-deviation through a scaling of the M values.

By using a FDR (false discovery rate) method in *limma*, corrections adjust the p value so it reflects the frequency of false positives. If 10 000 genes were to be tested and with a raw p value of 0.05, 500 genes would be expected as false positives. Of these 500 genes an adjusted FDR p value of 0.05, would only give 25 false positives. The reason for using an adjusted p value of 0.1 as a cut off (instead of $p \leq 0.05$) in the present study, is due to the minimal available information on detecting cellular responses at gene level after exposure to low doses of mixtures. Therefore, to have

openness to the material in the present study it was decided to use 0.1 as a cut off p value.

A gene list containing M values, A values together with the raw and adjusted p values was computed and saved as an excel file. Genes were considered to be differentially expressed when induction or repression was equal or above 1.3-fold compared to control. Genes with an M value between 0.4 and -0.4 was extracted from the data set due to a low differentially expression ratio of red and green intensities. Furthermore, spots with an A value below 9.5 was also extracted in order to include genes with lowest background intensities; together with an adjusted p-value over 0.1 (false discovery rate of 10%) was also eliminated from the data set.

Gene annotation

The differently expressed genes were annotated by using a web-based program "The Database for Annotation, Visualization and Integrated Discovery" (DAVID) 2007, developed by Laboratory of Immunopathogenesis and Bioinformatics (LIB), SAIC-Frederick, Inc. (Maryland, USA) to provide bioinformatics support to the National Institute of Allergy and Infectious Diseases (NIAID), USA, (http://david.abcc.ncifcrf.gov/). Accession numbers of the genes were inserted into DAVID, which compared them to its database and generated a list of selected genes with names and known functions.

Pathway analysis

By using the software Ingenuity Pathways Analysis (IPA) version 5.1 (Redwood City, USA), which is only valid for human, rat and mouse data, the identified genes were given a human homologue ID by using DAVID along with UniGene in PubMed in order to identify pathways, molecular mechanisms or biological processes most relevant to the identifier lists. IPA finds information from the full text of the scientific literature, including information about genes, drugs, chemicals, cellular and disease processes, and signalling and metabolic pathways, which no other programs available for the zebrafish do. In order to fulfil the pathways, the IPA software inserts other

nodes, such as genes, proteins, enzymes or other factors related to the experimental data.

Verification of differentially expressed genes

In order to validate gene expression profiles it is common to analyze a number of differentially expressed genes by quantitative real-time polymerase chain reaction (Q RT-PCR), which quantify your DNA after each round of amplification. Due to lack of time, this was not achieved. However, the zebrafish microarray platform used in the present study is well characterized and has been used for several years in this laboratory. Each time the experimental set up worked perfectly fine. From previous projects the verification by Q RT-PCR has shown an overall 95% correlation with the gene expression profiling, indicating that the result obtained in this study is reliable. Furthermore, the microarray data obtained from the female fish in the ZEBPOP project has been prioritized for confirmation by Q RT-PCR. These data showed good agreement with gene expression profiling, which would support the notion that the microarray data obtained on males in the present study are to be trusted.

Biomarkers of POPs exposure

Detection of biomarkers (EROD and Vitellogenin) has been performed in order to look for measurable alterations in biochemical processes.

EROD

The method used for detecting EROD activity is described by (Jonsson et al., 2002). 50 male fish (10 from each group) were used to dissect out three gill arches per fish. The gill arches were transferred separately to 24 wells plates and rinsed for 5 min in 300 µl HEPES-Cortland (HC) buffer (Appendix 2), followed by 5 min in 300 µl "substrate buffer" consisting of HC buffer supplemented with 7-ethoxyresorufin (Sigma) and dicumarol (Appendix 3). After rinsing, substrate buffer was replaced with 300 µl fresh substrate buffer. Since ER is sensitive to light, the plates were

covered with aluminium foil during 1 hr incubation. The substrate buffers were transferred to microtubes and stored at -20 ºC until assayed.

Resorufin (Sigma) standard solutions were prepared from a stock solution (10.4 mg resorufin in 10 ml DMSO) by dilution in distilled water (dH_2O), giving a concentration range of 0 – 16.315 ng resorufin/ml dH_2O. 200 µl of standards (in duplicates) and samples (in triplicates) were added to three Fluoronunc 96-wells plates. The fluorescence was detected using wavelengths 530 nm (excitation) and 580 nm (emission) in a Victor plate reader (Perkin Elmer, Massachusetts, USA). EROD activity was calculated by the Workshop software. The data was then transferred to excel and a new standard curve was made by using the mean of the three analysed standards. The mean values of the three gill arches were then used to manually calculate EROD activity via the standard curve. EROD activity was expressed as picomole of resorufin per gill arch per minute. Morten Sandvik, at the department of toxicology at the National Veterinary Institute, made the 7-ethoxyresorufin stock and the buffers.

Vitellogenin

For detecting the egg yolk precursor vitellogenin (Vtg) a commercial "Quantitative Zebrafish Vitellogenin ELISA test kit" (Biosense Laboratories, Bergen, Norway) was used, which is optimized and validated for blood and tissue samples from juvenile and male zebrafish. This ELISA is based on specific binding between antibodies and Vtg to quantify the protein in the samples. In order to perform the analysis, 50 frozen males (10 fish per exposure group), lacking gills, were homogenized with ultrathurrax for 5-10 seconds in homogenization buffer (Appendix 3), in 1:11 relationship of weight fish: buffer. After centrifuging for 1 hour at 12500 rpm at 4ºC, the homogenate (water phase) below the fat layer was transferred to cryo tubes and kept on ice until freezing at -80ºC.

Vtg assay procedure

The assay procedure for preparations and analysis of Vtg were performed in accordance with the manufacturers' instructions. The whole body homogenate

samples were diluted in order to hit the linear part of the standard curve. Initially one plate was run with five samples (one from each group) in four dilutions (1:11, 1:550, 1:550 000, 1:1 650 000) to find the most appropriate dilutions. Subsequently all the samples were diluted based on the first run.

Dilutions of standard and samples

The content of one vial of zebrafish Vtg standard (4.5 µg) was dissolved in 1 ml cold Dilution buffer, giving a Vtg stock solution of 4.5 µg/ml. The first dilution step for the standard curve was prepared by diluting 50 µl of the stock solution in 1750 µl cold Dilution buffer, giving a solution of 125 ng Vtg/ml. A two-fold serial dilution, 500 µl Vtg dilution + 500 µl buffer, including 11 steps was made, ending a concentration of 0.12 ng Vtg/ml. Samples were only analysed with one dilution in the second run with one of these dilutions 1:220 (25 µl sample to 475 µl buffer), 1:550 (10 µl sample to 490 µl buffer), 1:1650 (10 µl sample to 1490 µl buffer) and 1:2200 (5 µl sample to 995 µl buffer). Dilutions were kept on ice until use.

Incubation with standard and diluted samples

100 µl of Dilution buffer was added to each of the two NSB (non specific binding) wells. 100 µl of each Vtg standard dilution and of each sample dilution was added in duplicates. The plates was sealed and incubated at room temperature (20-25ºC) for 1 hour.

Incubation with Detecting antibody

The detecting antibody was diluted 1:350 by adding 31 µl to 11 ml Dilution buffer for each plate run in the assay. Plates was washed three times with 300 µl Washing buffer per well. 100 µl of the prepared antibody solution was added to all wells. The plates was covered and incubated at room temperature for 1 hour.

Incubation with Secondary antibody

The secondary antibody was diluted 1:2000 by adding 6 µl to 12 ml Dilution buffer for each plate run in the assay. The plates was again washed three times with 300 µl

Washing buffer per well before 100 µl of Secondary antibody solution was added to all wells. Plates were sealed and incubated at room temperature for 1 hour.

Development

Substrate solution was prepared just prior to use. Plates were washed five times with 300 µl Washing buffer per well, after which 100 µl Substrate solution was added. For 30 minutes the plates were incubated in room temperature in the dark. The reaction was stopped by adding 50 µl 2M H_2SO_4 to each well. After five minutes the absorbance was measured at 490 nm in a Victor plate reader (Perkin Elmer, Massachusetts, USA). By using a protocol made in the Workshop software it automatically calculated the means of the absorbance, and the concentration (ng/ml) of standards and samples. Since the samples were diluted the computed concentrations had to be multiplied by the dilution factor.

3.6 Statistical methods

Statistical analyses were performed using the JMP 6.0 software from SAS (SAS Institute, Cary, NC, USA). Data of the EROD and Vtg measurements were analyzed using one-way analysis of variance (ANOVA) while the body mass and length data were analyzed using three-way ANOVA, correcting for gender and purpose (due to a time difference in the sampling of fish; between 161 and 170 dpf). A two way ANOVA was performed with body mass and body length as dependent variables and exposure group and year of exposure as independent variables in order to investigate exposure effects across generations. The sex distribution and differences in sex ratio between years was analyzed using Chi-Square tests.

Additionally, differences between mean values were assessed with the Student's t-test. P-values<0.05 were considered significant.

The survival analysis was performed with the Kaplan Meier method, which calculates survival proportion at each observed time point. Survival differences for comparable groups across years (2006 and 2007) was investigated with multivariable survival

analysis using Cox's proportional hazards regression (Collett, 2001;Kleinbaum, 2000). The results are presented by proportional Hazard Ratio. The hazard ratio compares survival (time to death) in the exposed groups versus the unexposed control groups. A hazard ratio > 1 is predisposing and implies an increased risk, and a hazard ratio < 1 is preventive and implies an inverse association.

Survival analysis was also used to investigate the relationship between exposure and time to onset of puberty; the variable survival time was constructed as the number of weeks from the day of fertilisation until onset of puberty. Exposure groups (n=5), were compared with controls within year using the Log-Rank test. Censoring appeared when no egg laying had been observed between week 15 and 23 after fertilisation (2006: n=1; 2007: n=8). Onset of puberty was analysed for comparable groups across years (2006 and 2007) with multivariable survival analysis using Cox's proportional hazards regression (Kleinbaum, 2000;Collett, 2001). The results are presented by proportional Hazard Ratio. The hazard ratio compares time to onset of puberty in the exposed groups versus the unexposed control groups. The hazard ratio is explained above. Exposure group and year of study were explanatory variables.

For identification of differentially expressed genes the statistical software R version 2.5.1 was used (http://www.R-project.org/) (R foundation for statistical computing, 2004) with the *limmaGUI* package of Bioconductor (http://www.bioconductor.org/). Genes were considered to be differentially expressed with an adjusted p-value < 0.1. Associated genes were further mapped into signalling pathways by using the software Ingenuity Pathways Analysis (IPA).

3.7 Ethical aspects

The study was performed following the provisions enforced by the National Animal Research Authority. All partners were allowed from national authorities to work with the chosen laboratory animals and test the specific compounds.

4. Results

4.1 Chemical analysis of POPs

The concentration of POPs was analysed only in the parent generation and included HCB, HCHs, chlordanes, DDTs, PCBs, BDEs and HBCD. Dioxins and dioxin-like PCBs were not measured.

Concentrations of POPs in brine shrimp

The concentrations of OCs and BFRs in brine shrimp after 24 hours exposure are given in Table 4.1. The controls contained low levels of the listed compounds, ranging between n.d. – 17.8 ng/g lw for BDEs, n.d. – 6.4 ng/g lw for PCBs and 5.4 - 12 ng/g lw for DDTs. Dominating pollutants for the treatment groups were BDEs, especially BDE-47, DDE and PCBs (Table 4.1). The measured concentrations of toxins in brine shrimp exposed to three dose levels of Mjøsa mixture demonstrated a gradual increase in measured concentrations with increased exposure level [BDE 47: of 195 (low), 653 (medium), 3827 (high); ng/g lw, respectively].

Losna exposed brine shrimp showed pollutant levels in the same concentration range as the Mjøsa Low exposed group. However, the Losna group contained more DDTs, PCB-153 and a few other PCBs but lower levels of BDEs compared to the Mjøsa Low exposed group, i.e. 20.8 ng/g lw of PCB-138 in Losna contrary to 16.6 ng/g lw of PCB-138 in Mjøsa Low.

The HCHs and chlordanes were not detected in brine shrimp exposed to any of the three Mjøsa mixtures and low levels were found in the Losna exposed group (Table 4.1).

Table 4.1: *Measured concentrations (ng/g lipid weigh) of HCB, HCHs, chlordanes, DDTs, PCBs, BDEs and HBCD and fat percentages in 1 gram brine shrimp after 24 hours exposure during hatching. Detection limits given in section 3.1.*

	Brine shrimp				
	Control	**Losna**	**Mjøsa Low**	**Mjøsa Medium**	**Mjøsa High**
Lipid %	1.96	2.15	2.06	2.12	1.57
Component					
HCB	9.7	18.2	14.9	14.8	25.0
HCHs					
α-HCH	1.1	1.4	n.d.	n.d.	n.d.
β-HCH	1.1	1.6	n.d.	n.d.	n.d.
γ-HCH	3.0	1.3	n.d.	n.d.	n.d.
Chlordanes					
oxy-chlordane	0.3	0.6	n.d.	n.d.	n.d.
cis-chlordane	0.5	2.0	n.d.	n.d.	n.d.
trans-nonachlor	3.4	7.0	n.d.	n.d.	n.d.
DDTs					
pp-DDE	5.4	27.7	28.5	58.7	431.0
pp-DDD	12.0	26.3	21.1	23.7	73.1
pp-DDT	5.5	13.5	9.3	23.7	177.0
ΣDDTs	22.9	67.5	58.9	106.1	681.1
PCBs					
PCB-28	0.7	8.8	n.d.	n.d.	38.5
PCB-52	2.7	5.7	n.d.	n.d.	n.d.
PCB-47	n.d.	n.d.	n.d.	n.d.	n.d.
PCB-74	0.8	1.9	n.d.	n.d.	n.d.
PCB-66	0.8	3.6	n.d.	n.d.	n.d.
PCB-101	4.2	14.2	35.0	54.5	192.0
PCB-99	2.2	6.6	15.0	22.0	76.9
PCB-110	2.8	15.5	5.7	15.0	112.0
PCB-151	0.6	1.6	n.d.	n.d.	n.d.
PCB-149	2.9	8.3	n.d.	n.d.	n.d.
PCB-118	0.9	11.0	11.6	16.7	104.0
PCB-153	6.4	35.5	33.8	56.3	254.0
PCB-105	1.2	4.2	3.7	6.2	38.5
PCB-141	0.8	2.4	n.d.	n.d.	n.d.
PCB-137	1.5	3.1	n.d.	n.d.	n.d.
PCB-138	4.0	20.8	16.6	31.3	212.0
PCB-187	1.2	2.8	2.8	4.0	n.d.
PCB-183	1.5	1.8	n.d.	n.d.	n.d.
PCB-128	1.0	5.9	3.4	5.0	46.2
PCB-156	0.8	2.9	n.d.	n.d.	19.2
PCB-180	2.5	1.5	n.d.	n.d.	19.2
PCB-170	0.9	9.0	8.7	15.0	61.5
PCB-196	n.d.	2.6	3.7	5.0	26.9
PCB-189	n.d.	n.d.	n.d.	n.d.	n.d.
PCB-194	0.3	0.7	n.d.	n.d.	n.d.
PCB-206	n.d.	n.d.	n.d.	n.d.	n.d.
ΣPCBs	40.7	170.4	139.9	231.0	1200.9
BDEs					
BDE-28	n.d.	n.d.	n.d.	n.d.	48.1
BDE-47	17.8	30.4	195.0	653.0	3827.0
BDE-100	3.9	5.5	31.8	96.7	467.0
BDE-99	9.3	16.7	69.6	218.0	975.0
BDE-154	1.0	1.5	10.8	26.7	110.0
BDE-153	1.0	1.8	8.8	25.0	108.0
ΣBDEs	33.0	55.9	316.0	1019.4	5535.1
HBCD	n.d.	n.d.	n.d.	n.d.	908.0

Note; n.d. = not detected

Concentrations of POPs in zebrafish

After approximately five months of exposure the fish were chemically analysed for OCs and BFRs and the results are outlined in Table 4.2.

Toxins measured in the control group were generally low, ranging from 0.9 – 51.8 ng/g lw for BDEs, n.d. – 17.1 ng/g lw for PCBs and 2.1 – 21.6 ng/g lw for DDTs. The concentrations of POPs measured in zebrafish were generally lower than what was found in burbot liver from Lake Losna and Lake Mjøsa (Table 4.2; Appendix 1). However, almost all measured compounds from brine shrimp biomagnified in zebrafish. The most dominating pollutant, BDE-47 (marked in Table 4.2), showed a mean concentration of 5595.8 ng/g lw in Mjøsa High exposed fish, and 388.6 ng/g lw and 1155.5 ng/g lw of BDE-47 in Mjøsa Low and Mjøsa Medium exposed groups, respectively, whereas Losna exposed fish contained 93.3 ng/g of BDE-47. These concentrations indicated a 1.5-fold elevation of BDE-47 in Mjøsa High exposed fish and a 3-fold elevation in Losna exposed fish compared with levels found in brine shrimp. Interestingly, BDE-99 showed a decrease in all treatment groups from brine shrimp to zebrafish, indicating a possible metabolization of this compound in zebrafish. Mjøsa High exposed brine shrimp were found to contain 975 ng/g lw of BDE-99 contrary to 38.3 ng/g lw in zebrafish. The same was observed for HBCD. In Mjøsa High exposed brine shrimp the concentration of HBCD was 908 ng/g lw compared with 279.6 ng/g lw in zebrafish.

The most dominating components among the OCs found in zebrafish were PCB-153, PCB-138 and DDE. These compounds did not biomagnify that much as BDE-47 between brine shrimp and zebrafish (Table 4.2). For instance, concentrations of PCB-153 in brine shrimp exposed to low, medium and high dose levels of Mjøsa mixture contained 33.8, 56.3 and 254 ng/g lw of PCB-153 compared to 28, 65 and 313.9 ng/g lw of PCB-153 in zebrafish respectively. Losna exposed fish were found to contain slightly higher levels than Mjøsa Low exposed fish (Table 4.2). HCB levels in zebrafish were lower than in brine shrimp. The HCHs were not detected in any groups and chlordanes levels were generally low.

Table 4.2: *Measured concentrations (ng/g lipid weight) of HCB, HCHs, chlordanes, DDTs, PCBs, BDEs and HBCD and lipid percentages in the zebrafish after five months of exposure. Concentrations are presented as mean of pooled samples; 3 females and 3 males, whereas only 3 males from control fish. Detection limits are given in Section 3.1.*

	Zebrafish				
	Control	**Losna**	**Mjøsa Low**	**Mjøsa Medium**	**Mjøsa High**
Lipid%:	12.4	11.1	12.9	11.3	13.2
Component					
HCB	11.4	11.9	9.9	11.0	15.4
HCHs					
α-HCH	n.d.	n.d.	n.d.	n.d.	n.d.
β-HCH	n.d.	n.d.	n.d.	n.d.	n.d.
γ-HCH	n.d.	n.d.	n.d.	n.d.	n.d.
Chlordanes					
oxy-chlordane	0.8	1.2	0.6	0.7	n.d.
cis-chlordane	1.7	2.0	1.5	1.6	3.2
trans-nonachlor	7.0	8.8	6.6	8.8	16.8
DDTs					
pp-DDE	14.1	50.8	32.2	79.0	536.8
pp-DDD	21.6	24.7	17.3	19.6	41.5
pp-DDT	2.1	5.8	3.6	7.8	40.1
ΣDDTs	37.8	81.2	53.1	106.4	618.4
PCBs					
PCB-28	4.6	6.5	4.3	7.2	16.2
PCB-52	4.9	5.6	4.4	4.1	10.8
PCB-47	1.2	1.8	1.2	1.9	5.6
PCB-74	1.2	3.6	1.6	3.0	17.2
PCB-66	1.7	5.5	2.1	4.0	24.6
PCB-101	11.2	17.3	13.2	20.0	67.6
PCB-99	3.7	8.1	5.2	10.0	51.9
PCB-110	14.7	22.8	16.0	26.6	117.4
PCB-151	3.1	5.3	3.3	4.4	16.7
PCB-149	9.7	10.7	9.3	11.3	25.9
PCB-118	4.8	17.4	9.4	21.0	127.5
PCB-153	17.1	48.1	28.0	65.0	313.9
PCB-105	2.1	5.7	3.5	8.6	51.9
PCB-141	2.3	3.8	2.5	3.6	14.7
PCB-137	1.3	1.4	1.5	1.7	7.4
PCB-138	11.7	31.2	19.1	42.1	220.2
PCB-187	3.5	4.2	3.4	4.3	12.9
PCB-183	2.1	3.6	2.6	3.8	17.7
PCB-128	1.6	4.7	3.1	6.7	40.1
PCB-156	n.d.	4.2	2.5	4.9	26.6
PCB-157	n.d.	1.3	1.1	1.8	5.7
PCB-180	6.9	13.3	8.9	16.4	81.8
PCB-170	2.2	4.5	3.0	5.4	28.6
PCB-189	n.d.	0.7	0.4	0.5	2.3
PCB-194	1.1	1.3	1.0	1.7	5.6
PCB-206	n.d.	0.5	0.5	0.7	2.6
ΣPCBs	112.8	233.2	151.1	280.5	1313.4
BDEs					
BDE-28	3.6	3.9	11.6	26.2	98.5
BDE-47	51.8	93.3	388.6	1155.5	5595.8
BDE-100	6.9	11.3	39.7	113.6	536.2
BDE-99	0.9	0.8	1.9	6.3	38.3
BDE-154	2.1	3.0	9.8	23.0	113.4
BDE-153	1.6	2.0	9.0	21.8	106.0
ΣBDEs	66.9	114.4	460.7	1346.4	6488.2
HBCD	20.1	22.9	66.7	97.7	279.6

Note; n.d. = not detected

A comparison of some of the dominating POPs measured in burbot liver from Lake Mjøsa together with levels in brine shrimp and zebrafish after exposure to Mjøsa High for five months are illustrated in Figure 4.1. This clearly shows the dominance of BDE congeners in burbot from Lake Mjøsa.

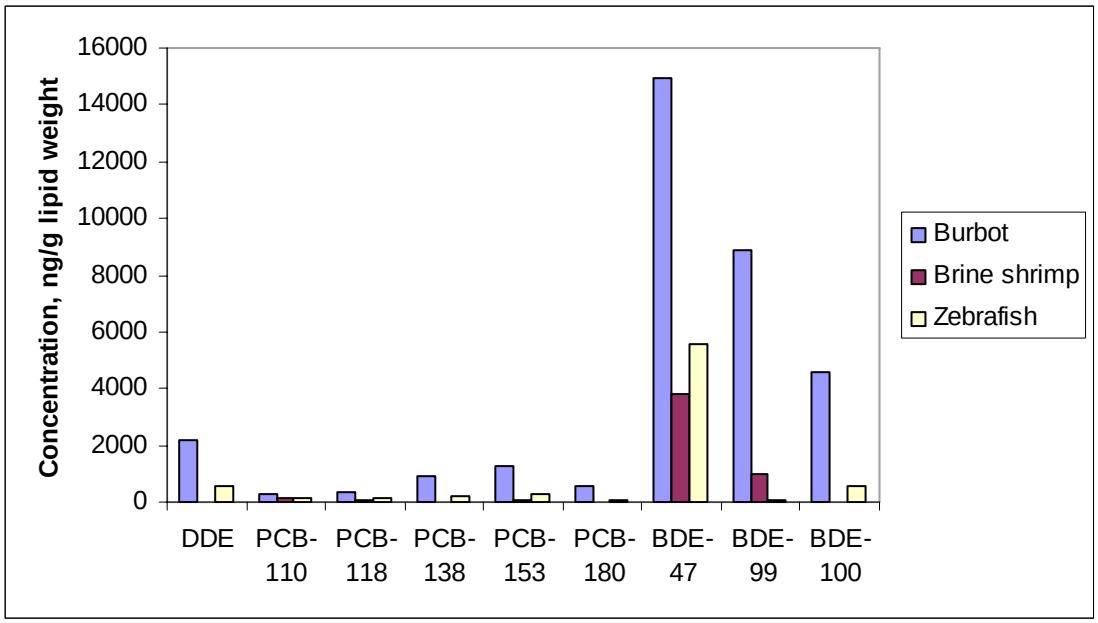


Figure 4.1: *Mean concentrations (ng/g lipid weight) of DDE, PCB-110, PCB-118, PCB-138, PCB-153, BDE-47, BDE-99 and BDE-100 in burbot liver from Lake Mjøsa, and brine shrimp and zebrafish exposed to Mjøsa High mixture.*

4.2 Demographic endpoints

Results from the demographic variables include survival, sex distribution, onset of puberty as well as growth of the fish.

Survival

F0 generation

A survival plot of the parent generation during 6 – 30 dpf is presented in Fig. 4.2. From 9-17 dpf there was a marked drop in survival in all groups of zebrafish. However after 20 dpf the survival rate stabilized between 70 – 77% in all groups (Fig. 4.2). Compared with the control group the Losna and Mjøsa High exposed groups had significantly lower survival ($p<0.04$ and $p<0.02$ respectively). The survival rates in the Mjøsa Low and Mjøsa Medium exposed groups were intermediate between Mjøsa High and Control and were not significantly different from controls ($p>0.05$).

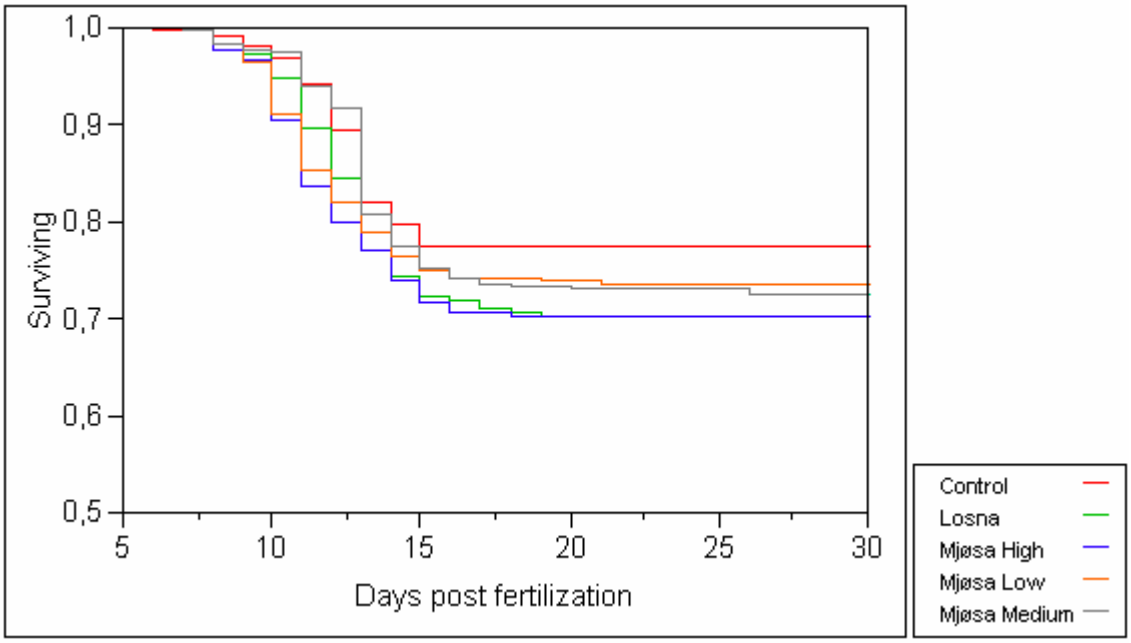


Figure 4.2: *Kaplan Meier survival plots covering the period 6-30 dpf for all experimental groups (colour-coded) in the F0 generation. Losna and Mjøsa High exposed fish had a significantly lower survival than Control fish ($p<0.05$).*

F1 generation

Offspring from the control group of the F0 generation had significantly higher survival than offspring from Mjøsa High and Losna exposed groups before start of feeding at 6 dpf ($p<0.0001$; Fig. 4.3).

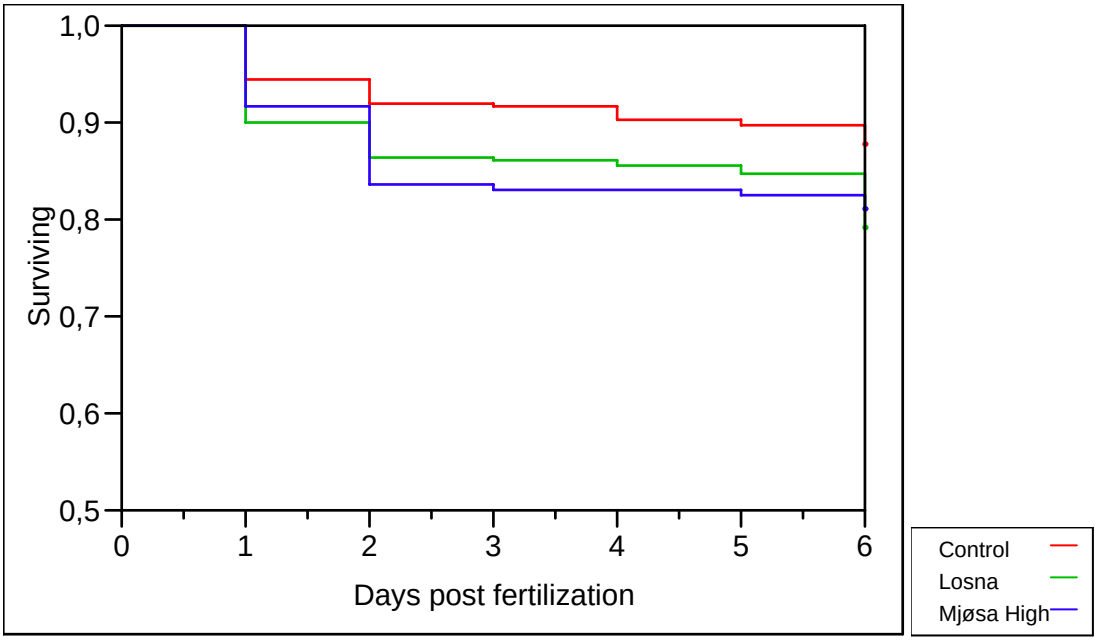


Figure 4.3: *Kaplan Meier survival plots for the early life stage (0 – 6 dpf) in the F1 generation. Each plot represents survival of offspring where their parents in the F0 generation had been allocated to the following exposure groups; Control (red), Losna (green) and Mjøsa High (blue). Losna and Mjøsa High exposed offspring differed significantly from the control offspring ($p<0.0001$).*

Kaplan Meier survival plots covering the time period from 7 – 30 dpf for the F1 generation are shown in Fig. 4.4. Similar to what was found in the F0 generation, all groups showed a significant decline in survival between 9-21 dpf, after which the mortality was very low.

The survival rates were generally lower in the F1 generation compared to the F0 generation (55-67% vs. 70 – 77%, respectively). Only in the Losna-Control and Mjøsa High-Mjøsa High groups the survival was significantly lower than in the

Control-Control group ($p<0.005$ and $p<0.014$, respectively; Fig. 4.4). Additionally, the Losna-Control group had significantly lower survival than the Losna-Losna and the Control-Losna groups ($p<0.03$ and $p<0.005$, respectively).

The Control-Mjøsa High group showed almost the same survival as fish exposed to the Mjøsa High treatment for two generations (Mjøsa High-Mjøsa High). The survival was significantly higher in the Losna-Losna group compared with the Mjøsa High-Mjøsa High group ($p<0.02$).

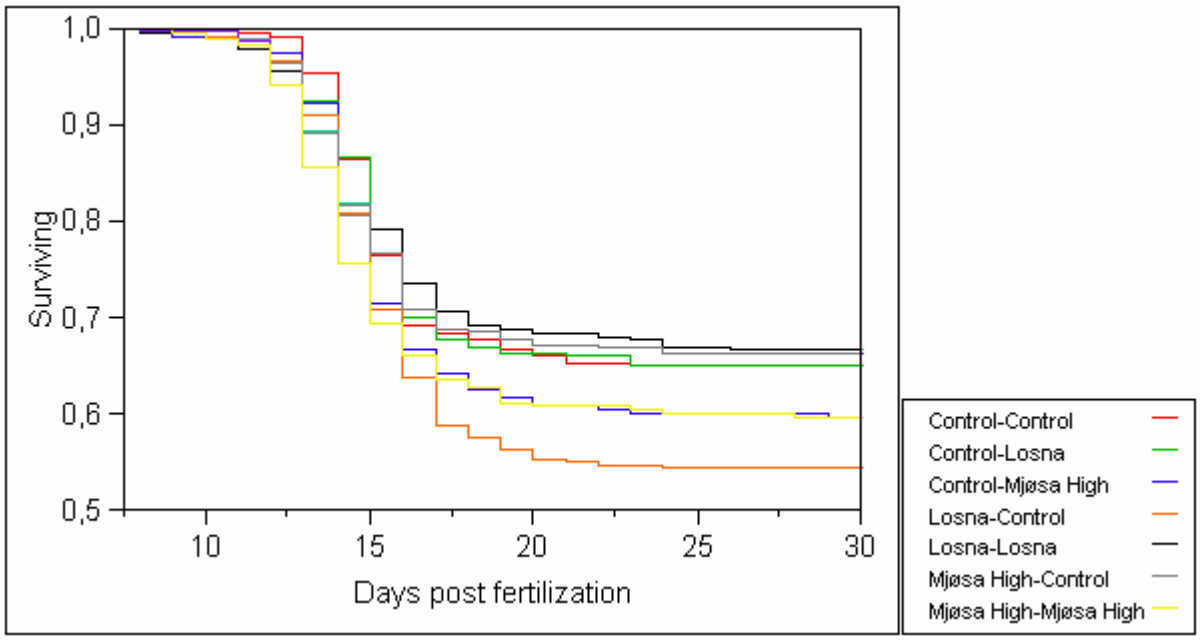


Figure 4.4: *Kaplan Meier survival plots covering the time period from 7-30 dpf for all groups (colour-coded) in the F1 generation. Each curve represents five replicates except the Control-Mjøsa High group which had only three replicates. Control-Control group showed a significantly higher survival than fish in Losna-Control and Mjøsa High-Mjøsa High exposed groups ($p<0.005$; $p<0.014$, respectively). Mjøsa High-Mjøsa High exposed fish showed a significantly lower survival than Losna-Losna exposed fish ($p<0.02$).*

The Cox proportional hazard model was run to investigate the relationship between survival and exposure across years for comparable groups related to Lakes Mjøsa and

Losna exposures. Control and Control-Control was analysed against Losna and Control-Losna, and Mjøsa High and Control-Mjøsa High, respectively.

An overall significant higher survival was found for the control groups in both models and year of study also contributed significantly to the variation in survival (Table 4.3).

Table 4.3: *The relationship between survival and the covariates exposure group and year of study, predicted by a Cox proportional hazard model.*

Variable	Hazard Ratio	β	SE	p-value*
Control	0,77	-0,26	0,044	<0,0001
Mjøsa High	.	.	.	.
Year 2006	0,77	-0,26	0,047	<0,0001
Year 2007	.	.	.	.
Control	0,90	-0,11	0,043	0,0085
Losna	.	.	.	.
Year 2006	0,86	-0,15	0,045	0,001
Year 2007	.	.	.	.

* Level of significance for the difference between categories of explanatory variables (Mjøsa High or Losna vs. Control, and Year 2007 vs. Year 2006).

Sex ratio

The results of the phenotypic assessment of gender after approximately five months of exposure in both generations are shown in Table 4.4 and Table 4.5. The overall phenotypic male/female sex ratio was 65/35 % in the F0 generation and 54/46% in the F1 generation. In the control groups the male/female sex ratios were 56.6/43.4% and 50/50%, respectively, in the F0 and F1 generations.

F0 generation

In the F0 generation there was an increase in the proportion of males in groups exposed to Losna and three dose levels of Mjøsa mixture compared to the control, (Table 4.4). However, only the Losna exposed fish had significantly more males (p= 0.033) compared with control fish. Taken together, Mjøsa exposed fish (Mjøsa High,

Mjøsa Low and Mjøsa Medium) had a male dominance which differed significantly from controls ($p<0.001$).

Table 4.4: *Mean phenotypic sex distribution of control zebrafish and groups of zebrafish exposed to real life mixtures of POPs extracted from Lake Mjøsa and Lake Losna for approximately five months in the F0 generation. Three doses of the Mjøsa extract were used (Low, Medium and High). The results are presented as mean percent of females and males of 5 replicates within each group. Chi-square test was used to assess group differences.*

Group	% females, mean	% males, mean	p-value*
Control	43.4	56.6	-
Losna	29.9	70.1	0.033
Mjøsa Low	32.7	67.3	0.093
Mjøsa Medium	34.0	66.0	0.144
Mjøsa High	33.3	66.7	0.105

* Level of significance for the difference between exposed groups and Control.

F1 generation

The sex distribution of fish in the F1 generation is shown in Table 4.5. Only the Mjøsa High-Mjøsa High group differed significantly from controls (more males; $p<0.02$). Furthermore, there was a significantly higher proportion of males in Mjøsa High-Mjøsa High and Mjøsa High-Control treated groups compared with Control-Mjøsa High exposed fish ($p<0.001$ and $p<0.02$ respectively).

Table 4.5: *Mean phenotypic sex distribution of control zebrafish and zebrafish exposed to real life mixtures of POPs extracted from Lake Mjøsa and Lake Losna for approximately five months in the F1 generation (Control-Control, Losna-Control, Mjøsa High-Control, Control-Losna, Losna-Losna, Control-Mjøsa High and Mjøsa High-Mjøsa High). The results are presented as mean percent of females and males of 5 replicates within each group. Chi-square tests were used to assess group differences.*

Group	% females, mean	% males, mean	p-value*
Control-Control	50.0	50.0	-
Control-Losna	49.8	50.2	0.964
Control-Mjøsa High	56.9	43.1	0.196
Losna-Control	45.1	54.9	0.310
Losna-Losna	45.0	55.0	0.293
Mjøsa High-Control	44.3	55.7	0.221
Mjøsa High-Mjøsa High	38.9	61.1	0.018

* Level of significance for the difference between exposed groups and Control-Control.

The differences in sex ratio between comparable groups in the F0 and F1 generations (Control versus Control-Control, Losna versus Control-Losna and Mjøsa High versus Control-Mjøsa High) are shown in Fig. 4.5. The sex ratio of the control groups from F0 and F1 generations were not significantly different. However, significantly more males were found in groups exposed to the Losna and Mjøsa High mixtures in the F0 generation than in the F1 generation ($p<0.0005$ and $p<0.0002$, respectively), indicating that year of study contributed significantly to the variation in sex ratio.

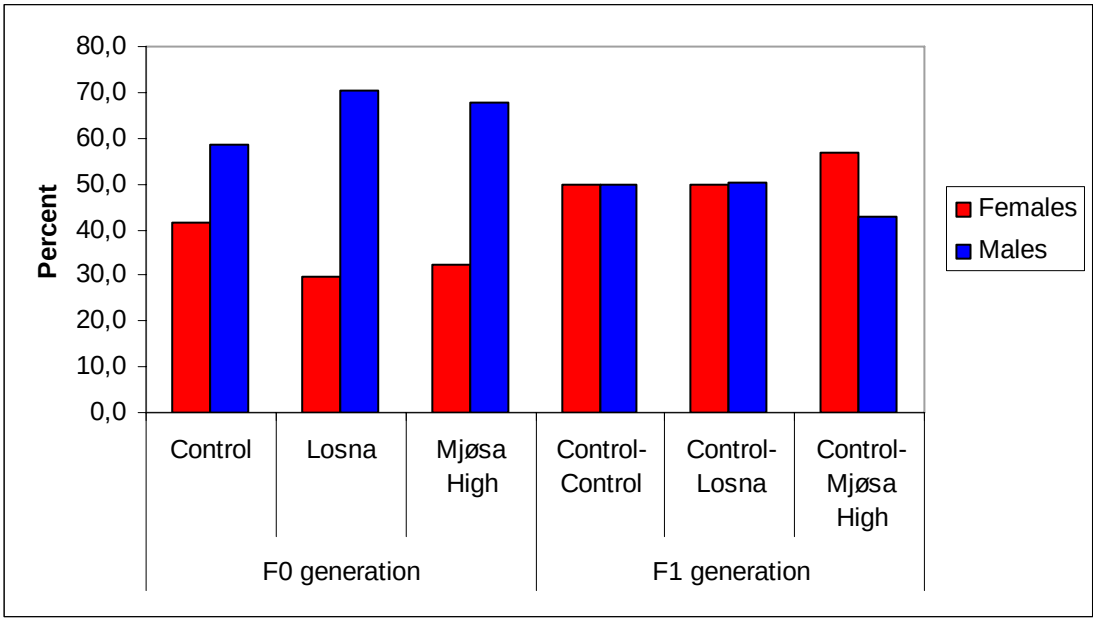


Figure 4.5: *Mean sex distribution (%) in comparable groups of zebrafish exposed to real life mixtures of environmental pollutants in two (F0 and F1) generations.*

Onset of puberty

Spawning was observed in 51 out of totally 60 replicates. In the F0 generation the overall mean observed time until onset of puberty was 20 weeks as compared to 18 weeks in the F1 generation. There was a general trend that control fish had a delay in the onset of puberty (Table 4.6).

Table 4.6: *Number of replicates (tanks) with observed spawning (n) and their mean (SE) time distance (weeks) from fertilization in two generations (F0: 2006 and F1:2007) in zebrafish exposed to environmental pollutant extracts. The time until first observed spawning within each exposure group was defined as onset of puberty.*

2006 (F0-generation)			
Group	**n**	**Mean, weeks**	**SE**
Control	5	21,4	0,4
Losna	5	19	1,8
Mjøsa Low	5	21,4	0,7
Mjøsa Medium	4	20	1,1
Mjøsa High	5	18,4	0,6

2007 (F1-generation)			
Group	**n**	**Mean, weeks**	**SE**
Control-Control	4	21	0
Control-Losna	3	17	1
Control-Mjøsa High	1	18	-
Losna-Control	4	18,5	1,2
Losna-Losna	5	17,4	0,6
Mjøsa High-Control	5	16,6	0,6
Mjøsa High-Mjøsa High	5	17,8	1

In the survival analysis of the 2006 (F0) data, the Mjøsa High group had a significantly earlier onset of puberty than the control group ($p<0.01$; Log-rank test). Similarly, the data from 2007 (F1) suggested that the Losna-Losna, Mjøsa High-Control and the Mjøsa High-Mjøsa High exposed groups differed significantly from the Control-Control group ($p\leq0.01$; Log-Rank test) in that they had an earlier onset of puberty.

When the Cox proportional hazard model was run to investigate the relationship between likelihood of onset of puberty and exposure across years for comparable groups related to Lake Mjøsa, a significant relationship was found between time to onset of puberty, and the explanatory variable year of study, but there was no effect of exposure (Table 4.7). No significant relationship was found between time to onset of puberty and the explanatory variables year of study and exposure for the Losna-exposed groups.

Table 4.7: *The relationship between time to onset of puberty [1)] and the covariates exposure group, year of study, predicted by a Cox proportional hazard model. The replicates (tanks) were observed either until confirmation of onset of puberty or censoring (n=8).*

Variable	**Hazard Ratio**	**β**	**SE**	**p-value***
Control	0.70	-0.36	0.32	0.26
Mjøsa High	.	.	.	.
Year 2006	0.55	-0.59	0.31	<0.05
Year 2007	.	.	.	.

[1)] Onset of puberty was defined as the week in which the first spawning was observed in each exposure group.
* Level of significance for the difference between categories of explanatory variables (Mjøsa High vs. Control and Year 2007 vs. Year 2006).

Total body mass and length

The mean body mass and length, for F0 and F1 generations of each exposure group are presented in Figs. 4.6 and 4.7, respectively.

F0 generation

Gender and exposure group contributed significantly to the variation in body mass. The overall mean body mass was 599.3 mg (range 235.6 – 1025.1 mg) in females and 436.1 mg (range: 244.3 – 761.4 mg) in males in the first generation. When correcting for gender, all treatment groups showed significantly ($p<0.0002$) higher mean body mass than controls.

The overall mean body length was 3.67 cm (range: 2.9 – 4.2 cm) in females and 3.57 cm (range: 2.7 – 4.9 cm) in males in the F0 generation.

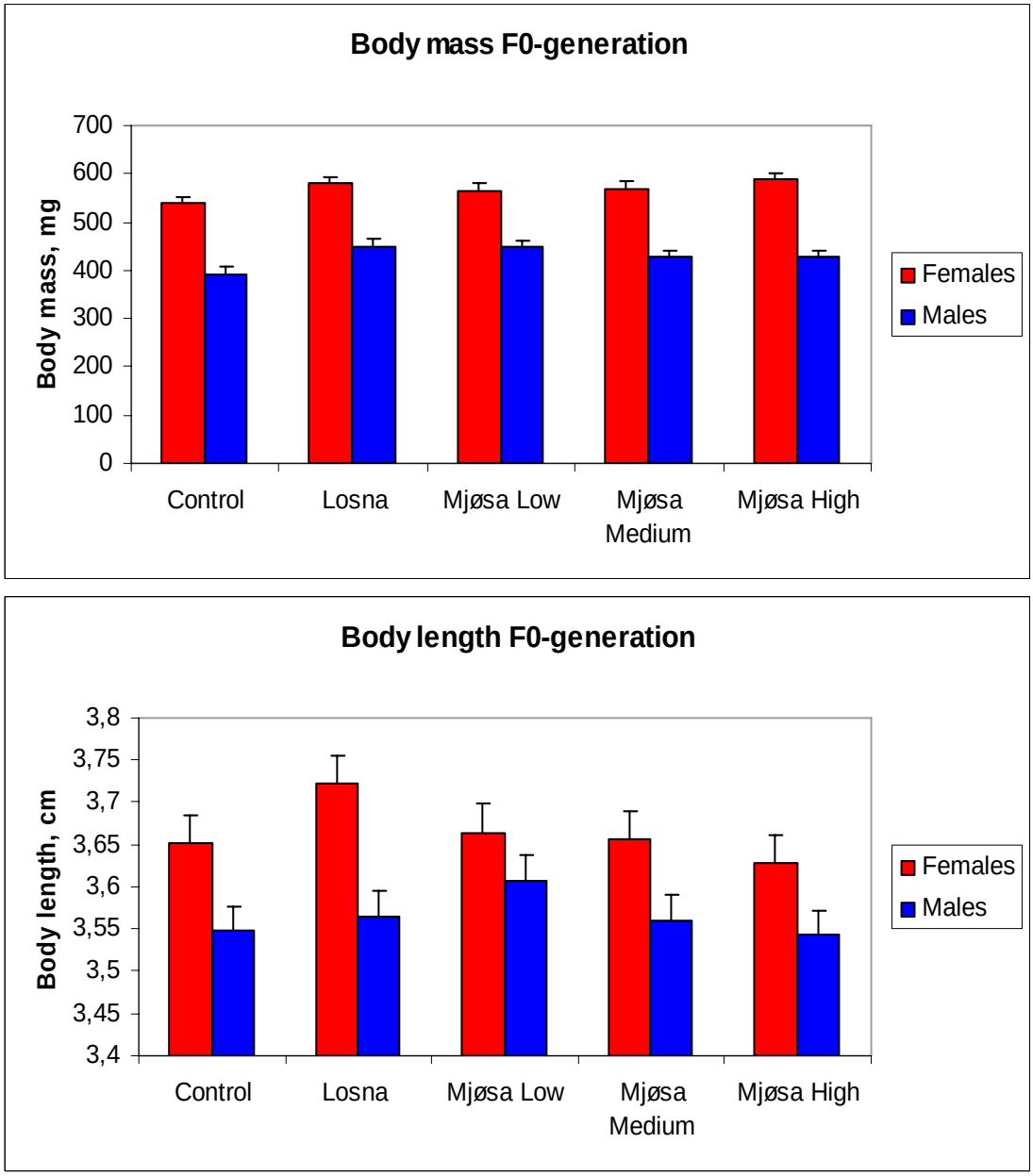


Figure 4.6: *Mean (±SE) body mass (a) and length (b) in the groups: Control, Losna, Mjøsa Low, Mjøsa Medium and Mjøsa High after approximately five months of exposure in the F0 generation. Each bar represent mean of five replicates [Control (n=115), Losna (n=120), Mjøsa Low (n=120), Mjøsa Medium (n=121), Mjøsa High (n=120)].*

F1 generation

The overall mean body mass in the F1 generation was 632.4 mg (range 251.6 – 1276 mg) in females and 476.5 mg (range: 249 – 806.9 mg) in males. Contrary to the body mass in the F0 generation, a significantly ($p<0.0001$) lower body mass was found in

exposed groups compared with unexposed groups (Control-Control and Losna-Control).

The overall mean body length was 3.84 cm (range: 3.1 – 4.5 cm) in females and 3.76 cm (range: 2.9 – 4.3 cm) in males. The body lengths of the Control-Control and Losna-Control groups were significantly longer than other groups ($p<0.002$).

Furthermore, a significant ($p<0.0001$) interaction was found between gender and exposure group, suggesting that the gender effect was not similar across exposure groups.

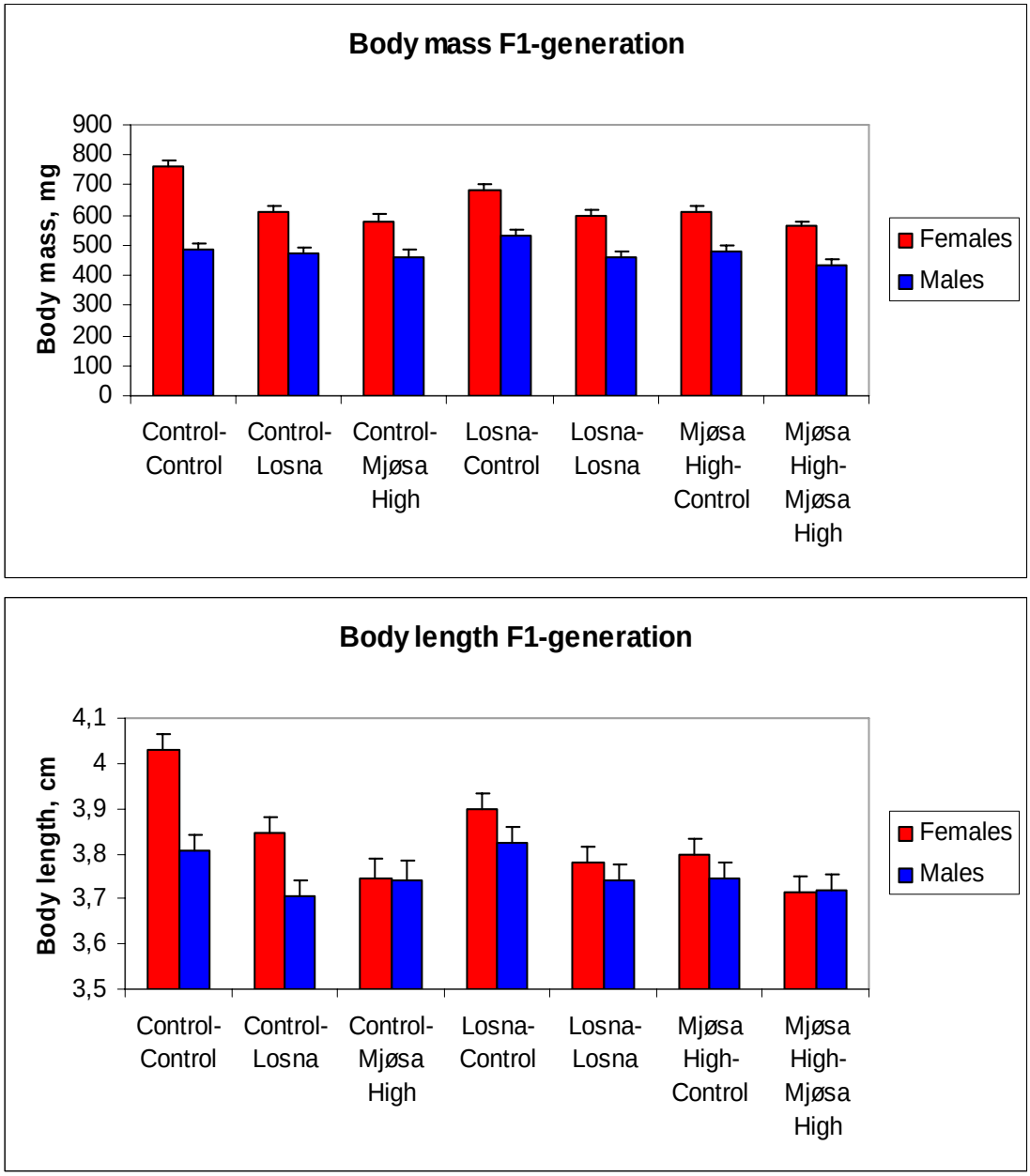


Figure 4.7: *Mean (±SE) body mass (a) and length (b) in the control and Losna and Mjøsa High exposure groups in different combinations after approximately five months of exposure in F1 generation. Each bar represents the mean of five replicates for n=150, except Control-Mjøsa High group where n=90.*

Differences in mean body mass and body length between comparable groups in the F0 and F1 generations were investigated (Table 4.8), by using two way ANOVA with body mass and body length as dependent variables and exposure group and year of exposure as independent variables.

Control fish in the F1 generation were significantly heavier and longer than those in the F0 generation ($p<0.0001$; Table 4.8). The mean body length of the Control-Losna exposed fish in F1 generation was significantly increased compared with Losna exposed fish in the F0 ($p<0.0001$). However, the mean body mass of Losna exposed fish was not significantly different in the two generations. The same was true for the body mass and length of the two Mjøsa High exposed groups.

Table 4.8: *Mean± SE body mass (mg) and body length (cm) of females and males in comparable groups (Control vs. Control-Control, Losna vs. Control-Losna and Mjøsa High vs. Control-Mjøsa High) in F0 and F1 generations respectively. The number of fish ranged from 115 – 150 between groups. Comparable groups with different superscript differ significantly ($p<0.05$), and comparable groups without superscript were not significantly different.*

		Body mass, mg		**Length, cm**	
		Females	**Males**	**Females**	**Males**
F0 generation	Control	556,2 ± 17,3[a]	400,8 ± 15,2[a]	3,66 ± 0,03	3,54 ± 0,03
	Losna	599,3 ± 11,4	455,9 ± 10,3	3,74 ± 0,03[c]	3,57 ± 0,02[c]
	Mjøsa High	600,5 ± 12,6	434,1 ± 11,6	3,64 ± 0,03	3,54 ± 0,03
F1 generation	Control-Control	760,5 ± 14,1[b]	486,9 ± 14,1[b]	4,03 ± 0,02	3,8 ± 0,02
	Control-Losna	609,5 ± 9,6	474 ± 9,6	3,85 ± 0,02[d]	3,71 ± 0,02[d]
	Control-Mjøsa High	580,6 ± 13,9	460,1 ± 13,9	3,74 ± 0,03	3,74 ± 0,03

Two-way analyses of variance were performed with body mass and body length as dependent variables and exposure group and year of exposure as independent variables. Year of exposure contributed significantly to the variation in both body mass and body length ($p<0.0001$).

4.3 Gene expression analysis

Gene expression analysis was performed on livers and testis from male fish exposed to Losna and Mjøsa High mixtures and compared with livers and testis from control males in the first generation. Design of the experimental model is shown in Fig. 3.3. In general, the effects detected at the gene level in the male zebrafish after exposure to environmental concentrations of real life mixtures (Mjøsa High) were associated to endocrine regulation, AhR signalling, endoplasmic reticulum stress, and apoptosis.

RNA quality results

Mean RNA concentrations isolated and purified from the harvested tissue material ranged between 404 – 552 ng/µl of RNA for testis samples and 894 – 1122 ng/µl of RNA for liver samples. The 260/280 nm ratio for the RNAs were over 2.1 for all samples.

Results from the bioanalyzer indicated that all samples were of high quality, with a mean 28S:18S ribosomal RNA ratio of 1.5, 1.4 and 1.7, for testis samples from control, Losna and Mjøsa High groups, respectively, and 1.5, 1.7 and 1.5 for the liver samples, respectively. Mean RIN numbers in control, Losna and Mjøsa High groups were calculated to be 8.6, 8.2 and 9.2 for the testis, and 9.2, 8.8 and 9.4 for liver samples, respectively. In addition the electropherogram showed no degradation or contamination of the RNA samples. An electropherogram with RNA of good quality of liver samples from Mjøsa High exposed males is presented in Fig. 4.8. All liver and testis samples showed also good RNA quality.

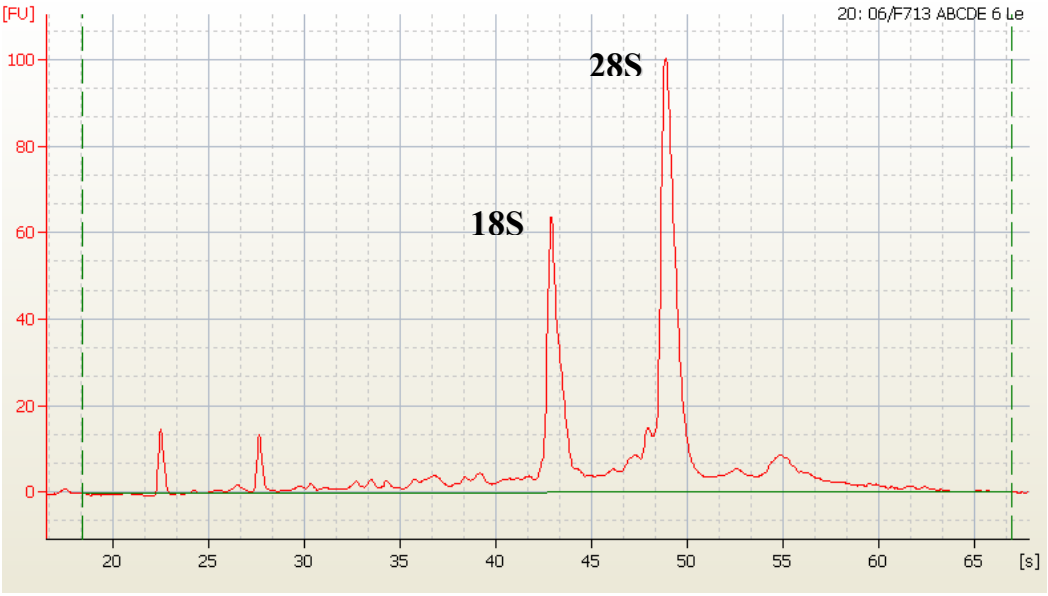


Figure 4.8: *An electropherogram of a RNA sample from livers of Mjøsa High exposed males by using Agilent 2100 Bioanalyzer. The X axis displays the integrity time (seconds) and y axis represents the fluorescence (FU; Fluorescence Unit). The ribosomal RNA peaks, 18S and 28S, are indicated in the diagram.*

Microarray results

In total, twenty four hybridizations were carried out, representing 6 hybridizations in each of four groups; Losna-Liver, Mjøsa High-Liver, Losna-Testis and Mjøsa High-Testis.

Normalization

Results from the normalization are presented as MA–plots by showing plots before and after normalization (Fig. 4.9).

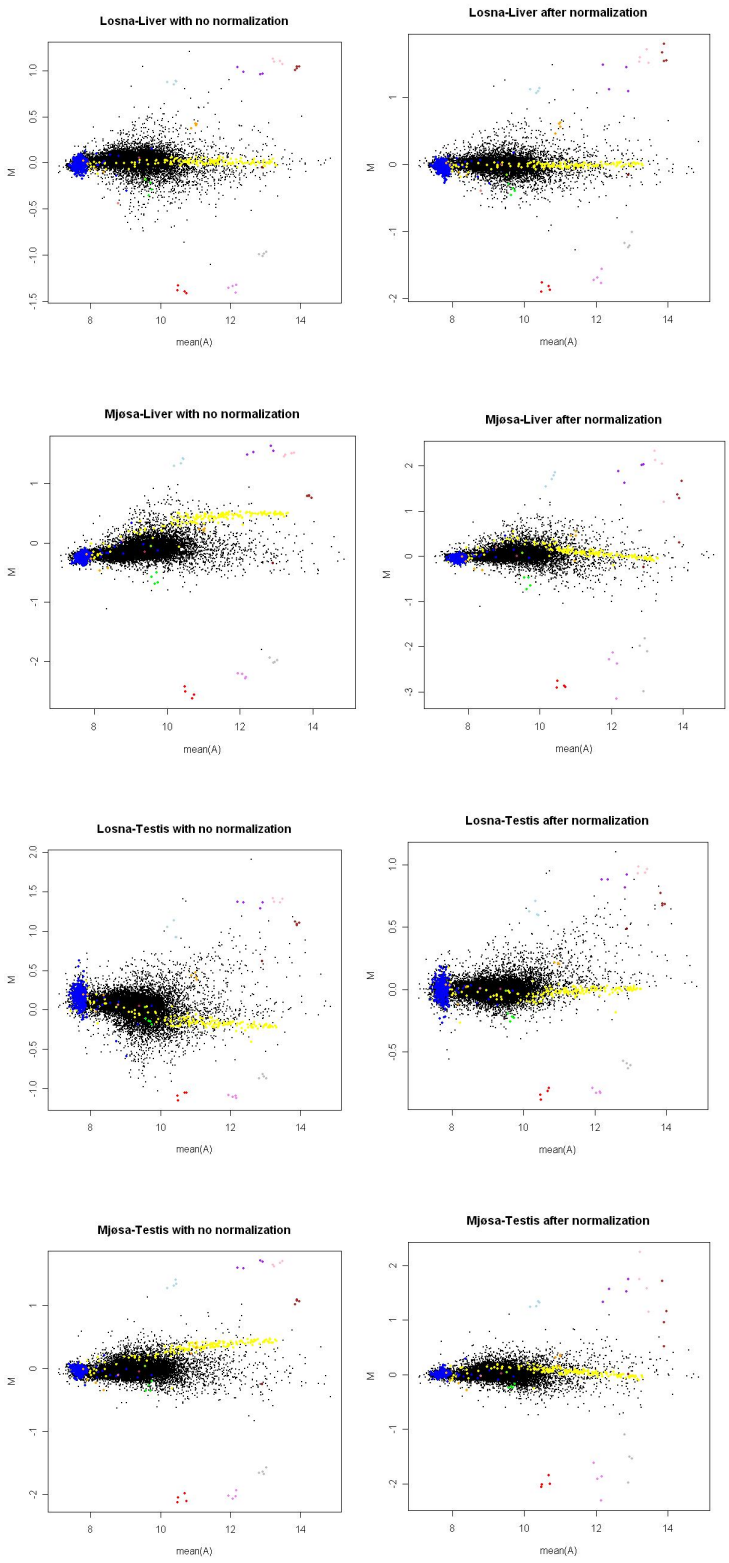


Figure 4.9: *Average MA-plots for four the groups before and after normalization; Losna-Livers, Mjøsa High-Livers, Losna-Testis and Mjøsa High-Testis. All groups have been compared to the control group. X axis displays the mean A value and y axis displays the fitted M value.*

Identification of expression patterns

Lists containing genes, differentially expressed for the exposed tissues were generated. Up- and downregulated genes were identified with name and function in DAVID. No significant differentially expressed genes in any of the tissues were identified in Losna treated males.

In contrast, microarray analysis of the Mjøsa High exposed zebrafish genome, revealed consistent significant changes in a total of 95 and 21 genes from the liver and testis, respectively.

Table 4.9 summarizes the total amount of differentially expressed genes.

Table 4.9: *Total number of differentially expressed genes in liver and testis of male zebrafish after approximately five months of exposure to Mjøsa High and Losna mixture. Amount of annotated genes in DAVID as well as unknown genes in the tissues are also presented.*

	Mjøsa High				**Losna**			
	Liver		**Testis**		**Liver**		**Testis**	
	Up	Down	Up	Down	Up	Down	Up	Down
Annotated	41	37	16	4	0	0	0	0
Unknown	12	5	1	0	0	0	0	0
Total	53	42	17	4	0	0	0	0

A list of the differentially expressed genes is presented in Table 4.10. Human homologue identifications are given where possible. However, a number of genes could not be assigned a name and/or a function. In order to find genes associated with common signalling pathways or cellular processes, IPA software analysis was used. Three pathways were established by IPA among the liver genes from fish exposed to Mjøsa High mixture using the human homologues. These are outlined in the

discussion (Section 5.3; Fig. 5.1 – 5.3) due to lack of experimental documentation for the zebrafish.

Table 4.10: *Differentially expressed genes from liver and testis tissue in Mjøsa High exposed fish. Genes are listed with gene name, human homologue, M value, fold change (2^M) as well as adjusted p value.*

Zebrafish Acc.no.	Gene name	Human homologue	M value	Fold change	Adj. P value
LIVER					
BI888210	Polypyrimidine tract-binding protein		1,133	2,193	0,022
AI545475	Angiotensin-converting enzyme	ACE	1,070	2,099	0,032
AW233227	Anterior gradient-like protein		0,844	1,794	0,067
BG303979	Thioredoxin reductase 1		0,746	1,678	0,008
AW058863	Solute carrier family 38, member 2 like	SLC25A16	0,725	1,653	0,049
BI673285	Phosphatidylinositol 3-kinase p55 subunit		0,708	1,634	0,025
AW175483	Cndp dipeptidase 2		0,674	1,595	0,091
BG891983	Arginase, type 2	ARG2	0,673	1,594	0,022
BI888604	Dual specificity phosphatase 1	DUSP1	0,662	1,582	0,077
AF064835	Translation elongation factor 2, like		0,661	1,581	0,039
BM185334	Nuclear receptor coactivator 4	NCOA4	0,640	1,558	0,028
AF304130	Roundabout homolog 1	ROBO1	0,637	1,555	0,016
BE016515	Inositol 1,4,5-trisphosphate 3-kinase		0,611	1,527	0,075
BG305824	Malate dehydrogenase	MDH2	0,550	1,464	0,082
BI879683	Serine/threonine-protein kinase	PIM1	0,516	1,430	0,067
AJ005692	Signal transduction and activation of transcription 1		0,502	1,416	0,035
AI437149	Integral membrane protein 1		0,456	1,372	0,047
AI641767	Tax1 binding protein 3		0,453	1,369	0,057
BM183630	Cyclic AMP phosphoprotein		0,452	1,368	0,022
BI896504	Ribosomal protein S23		0,444	1,361	0,039
BI886451	Nucleolar protein 5a	NOP5/NOP58	0,442	1,358	0,072
AF099738	Junction plakoglobin	JUP	0,432	1,349	0,008
AW232181	Integral membrane protein 1		0,416	1,334	0,065
AW280156	ADP-ribosylhydrolase like 2		0,412	1,331	0,079
BI892384	Heterogeneous nuclear ribonucleoprotein k		0,404	1,323	0,032
BI474990	Autocrine motility factor		-0,403	-1,323	0,013
AW279746	Kruppel-like factor 11		-0,429	-1,346	0,079
AA494790	Dicarbonyl/l-xylulose reductase		-0,435	-1,352	0,038
BM082666	Cytochrome c oxidase subunit va	COX5A	-0,445	-1,361	0,065
CF269307	Stress-associated endoplasmic reticulum protein	SERP1	-0,445	-1,361	0,065
BI891138	Ribosomal protein L35a		-0,447	-1,363	0,051
AI497455	Hydroxysteroid dehydrogenase like 2	HSDL2	-0,464	-1,379	0,096
BI891769	Ribosomal protein l10a		-0,468	-1,383	0,095
BI885824	Endothelial differentiation-related factor 1	EDF1	-0,469	-1,384	0,072
BI476461	Sec24-related protein D	SEC24D	-0,478	-1,393	0,051
BM036395	Vitellogenin 1		-0,488	-1,403	0,038
BE017206	Mitochondrial 18 kDa protein		-0,505	-1,419	0,060
BI326273	NADH-ubiquinone oxireductase precursor		-0,527	-1,441	0,087
BG303234	Prolyl hydroxylase domain containing protein	EGLN1	-0,550	-1,464	0,052
AI878489	Heat shock protein 22	HSPB8	-0,567	-1,482	0,083
BI878656	Coiled-coil domain containing 47		-0,607	-1,523	0,008
AI477544	Signal sequence receptor, alpha	SSR1	-0,607	-1,523	0,013
BI882727	Hydroxysteroid dehydrogenase like 2	HSDL2	-0,624	-1,541	0,028
AA494800	Calcium binding and coiled-coil domain 1	CALCOCO1	-0,631	-1,549	0,027
AI884210	Acyl-Coenzyme A dehydrogenase, very long chain		-0,675	-1,597	0,088
AW567144	60S ribosomal protein		-0,690	-1,613	0,038
AW342801	Cytochrome c oxidase assembly protein	COX10	-0,718	-1,645	0,040
BI842921	Ribosomal protein s10	RPL10A	-0,722	-1,649	0,079
AW154758	Protein tyrosine phosphatase-like, member b		-0,727	-1,655	0,038
BI843229	Translation initiation factor 1A		-0,783	-1,721	0,013
AW233688	Succinate-coa ligase, alpha subunit		-0,788	-1,726	0,010
BE557668	Translation elongation factor 1 beta 2		-0,791	-1,730	0,067
AW420304	Mixed Lineage Leukaemia		-0,796	-1,736	0,013
AW154620	ATP-dependent RNA helicase		-0,902	-1,868	0,033
AI964218	Ribosomal protein 7a	RPL7A	-1,030	-2,042	0,080
TESTIS					
BI888210	Polypyrimidine tract-binding protein	PTBP1	1,110	2,159	0,019

AW127912	Solute carrier family 25 alpha, member 5	SLC25A5	0,643	1,561	0,057
BI888721	Nucleophosmin 1	NPM1	0,610	1,526	0,064
L35587	Heat shock protein 90-beta	HSP90AB	0,585	1,500	0,016
BI888822	Golgi autoantigen 7	GOLGA7	0,572	1,487	0,043
AI964190	Reticulon 4a	RTN4	0,512	1,426	0,095
AI641717	Survival motor neuron domain containing 1	SMNDC1	0,479	1,394	0,046
AF041440	One-eyed pinhead	TDGF1	0,463	1,379	0,095
BI892155	Cold inducible rna binding protein	EEF1D	0,442	1,359	0,074
AW305541	Ring finger protein 2		0,422	1,340	0,037
BM095208	Solute carrier family 25, member 40		0,413	1,332	0,042
BI887554	Ubiquitin-conjugating enzyme E2A	UBE2A	0,411	1,330	0,092
BG304158	Set translocation (myeloid leukaemia-associated) b	PDCD10	0,408	1,327	0,079
AI958191	Lysyl-trna synthetase	KARS	0,401	1,320	0,005
BI889901	Pumilio homolog 1	PUM1	-0,445	-1,361	0,066
BI842921	Ribosomal protein s10	RPS10	-0,700	-1,624	0,088
BI878615	Calcium homeostasis endoplasmic reticulum protein	CHERP	-0,739	-1,669	0,095

4.4 Biomarkers

Results from biomarkers detection was only obtained from the first generation.

EROD

The mean EROD activity varied between groups from 0.054 to 0.069 pmol resorufin/gill arch/min (Fig. 4.10). The control group had significantly lower EROD activity than all exposed groups ($p<0.0001$). The highest EROD activity was found in the Mjøsa Low group (0.068 pmol resorufin/gill arch/min; Fig. 4.10).

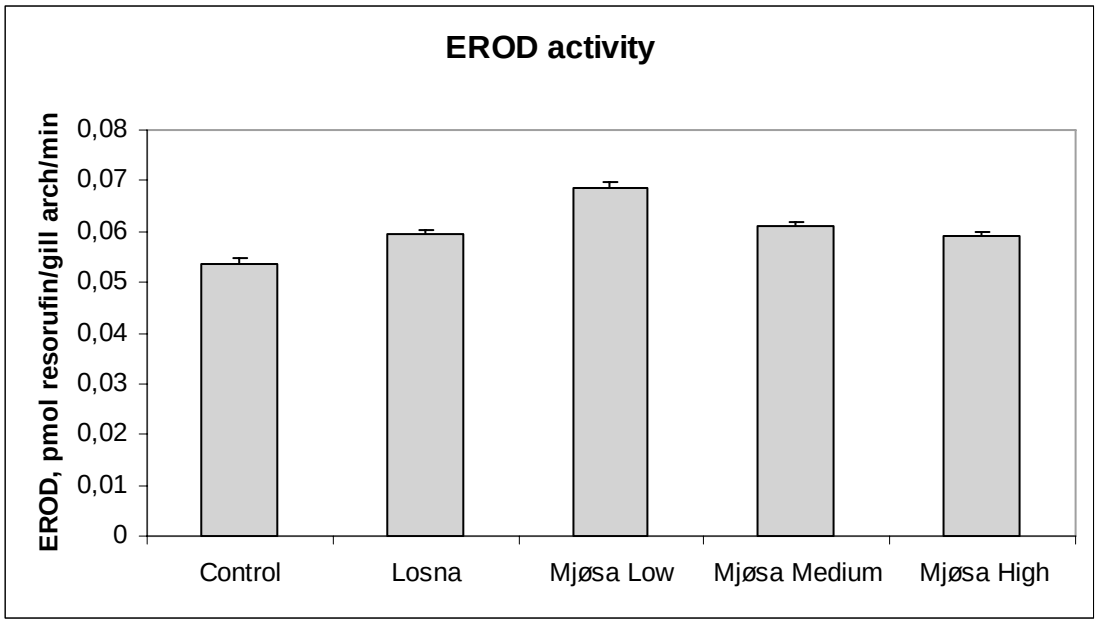


Figure 4.10: *Mean (+SE) EROD activity, expressed as pmol resorufin/gill arch/min, in zebrafish after five months of exposure to solvent control, Losna mixture and Mjøsa mixture in three dose levels. Control and Mjøsa Low differed significantly from all other groups ($p<0.0001$). Mjøsa Medium (n=28), Control and Mjøsa Low (n=29), Losna and Mjøsa High (n=30).*

Vitellogenin

Results from the whole body vitellogenin measurements from the ELISA test are given in Fig. 4.11. The mean Vtg concentration varied between groups, ranging from 9985 to 111716 ng Vtg/g.

Mjøsa Low treated fish had a significantly higher mean Vtg concentration compared to controls and other treatment groups. The lowest mean Vtg concentration was found in control fish (9950 ng/g ± 3171) while the highest concentration was found in the Mjøsa Low group (111716 ng/g ± 40022), indicating a 10 times increase in Vtg levels compared to control ($p<0.0001$). The mean Vtg concentrations in the Losna, Mjøsa Medium and Mjøsa High groups did not differ significantly from controls.

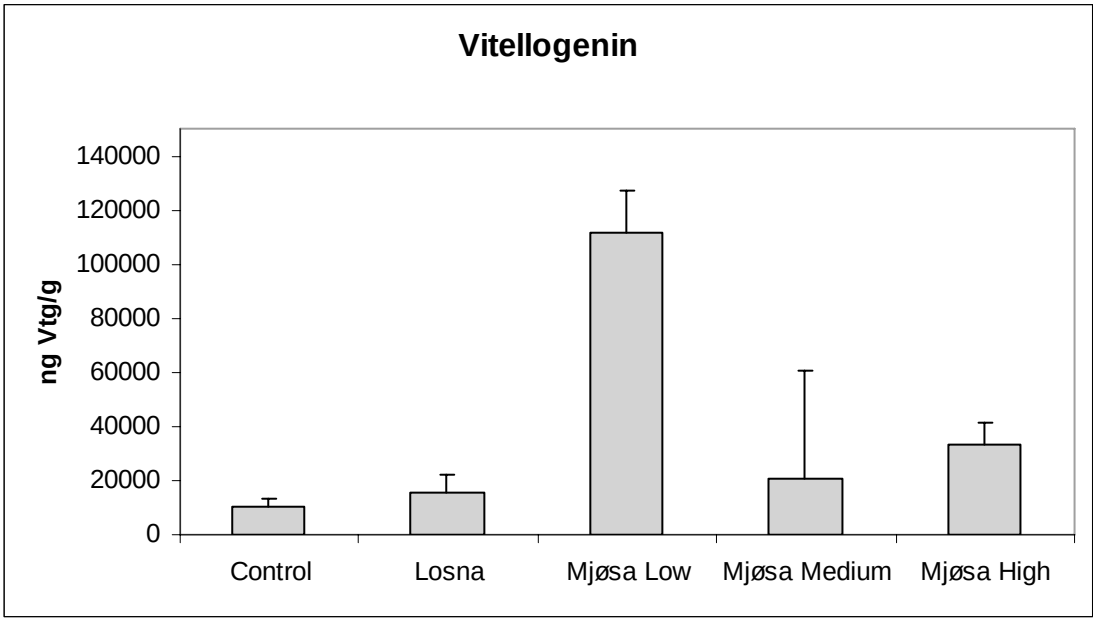


Figure 4.11: *Concentrations of vitellogenin (Vtg; ng/g) in whole body homogenate samples of control males and males exposed to Losna and Mjøsa mixtures for approximately five months of exposure. Mean ± SE, n=9 for control group, n=10 for treatment groups. Only Mjøsa Low differed significantly from Control (p<0.0001).*

5. Discussion

5.1 Biomagnification of POPs

The present study showed an overall biomagnification of POPs in zebrafish exposed to environmental relevant concentrations of natural mixtures harvested from Lake Mjøsa and Lake Losna. However, metabolism of some congeners was also indicated.

In burbot liver extracts from Lake Mjøsa, BDE-47, BDE-99, BDE-100, PCB-153, PCB-138, PCB-180 as well as *p-p*-DDT and its derivative *p-p*-DDE were the most abundant congeners (Appendix 1). This is in agreement with previous reports (Ulbrich and Stahlmann, 2004;Rice et al., 2002).

In contrast to the decreasing time trends of PBDEs in the Nordic countries, the result of the present study suggest that the levels are still increasing in Lake Mjøsa. In 2001, the concentration of ΣPBDE for BDE-47 and -99 detected in burbot liver ranged between 1500-3900 ng/g lw in 2001 (Fjeld et al., 2001). In 2003, NIVA measured a mean concentration of 15400 ng/g lw and three years later, in the present study, a concentration of 23807 ng/g lw of ΣPBDE was found for the same components (14917 and 8890 ng/g lw for BDE-47 and -99, respectively; Appendix 1). This would indicate a recent and/or ongoing pollution in Lake Mjøsa. Furthermore, the concentration of BDE-47 (used as marker for PBDE pollution in the refered studies) in the present study is remarkably high compared to fish from other regions of the world; 257 ng/g lw in California costal waters, 5-7000 ng/g lw in Swiss rivers, 63-307 ng/g lw in the North sea, 29-744 in Spain rivers and 2440 ng/g lw in Lake Michigan (Brown et al., 2006;Hartmann et al., 2007;Labandeira et al., 2007;Labandeira et al., 2007;Fjeld et al., 2004).

In the present study, the PCB levels in burbot liver from Lake Mjøsa (Appendix 1) were more or less similar to those reported by Fjeld et al., (2001). The same trend was found also for DDT in burbot liver; ΣDDT (*p-p*-DDT, *p-p*-DDE, *p-p*-DDD) was

ΣDDT 2200-7400 ng/g lw in 2001 depending on the capture location and 3280 ng/g lw in 2004 [Appendix 1; (Fjeld et al., 2004)].

The POP levels found in burbot liver from Lake Losna indicate that this is a relatively uncontaminated area compared to Lake Mjøsa. However, some pollutants such as DDE, PCB-153 and PCB-138, were found in moderate concentrations (Appendix 1).

Accumulation and metabolization of POPs in zebrafish

The three exposure dose levels of the Mjøsa mixture (low, medium and high) resulted in a wide range of tissue concentrations (388.6 - 5595.8 ng/g lw of BDE-47) in zebrafish (Table 4.2). The Mjøsa High exposed zebrafish contained 5595.8 ng/g lw of BDE-47, which is lower than in the burbot from Lake Mjøsa (14917 ng/g lw; Appendix 1), but higher than in trout from Lake Mjøsa [3300 ng/g lw; (Fjeld et al., 2004)]. This indicates that the levels obtained in the experimental fish are environmental relevant representing exposure levels in wild fish. These Mjøsa High levels are considerably higher than detected in human samples, i.e. 32 – 245 ng/g lw of BDE-47 in serum samples from a Californian family (Fischer et al., 2006) and 2.9 – 271.5 ng/g lw of BDE-47 in mothers milk from Texas (Schecter et al., 2003a). Blood levels of PBDEs of people with high intake of fish from Lake Mjøsa were found to contain between 0.30 – 38 ng/g lw of BDE-47 (Thomsen et al., 2006). However, these levels are in the same range as detected in zebrafish exposed to Losna mixture as well as the lowest Mjøsa dose.

The PCB levels obtained in the present study represents also levels found in human samples. PCB-153 was detected to 48.1, 28, 65 and 313.9 ng/g lw for Losna, Mjøsa Low, Mjøsa Medium and Mjøsa High exposed fish, respectively. Levels ranging between 77 – 130 ng/g lw of PCB-153 has been found in milk samples from Italian women (Ingelido et al., 2007), and mothers living in Poland was found to contain 4.8 – 43.7 ng/g lw of PCB-153 in maternal serum (Jaraczewska et al., 2006).

Results from a study with medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*) fed BDE-47 exposed *Artemia,* suggests that BDE-47 was well absorbed from the gastrointestinal tract and that the fish had limited capacity to excrete BDE-47 (Muirhead et al., 2006). This demonstrates that *Artemia* is a reliable way of exposing the zebrafish. Furthermore, a study conducted on juvenile carp (*Cyprinus carpio*) exposed to a mixture of four BDE congeners (BDE-28, BDE-47, BDE-99 and BDE-153) through their diet for 60 days, showed high absorption efficiencies for BDE-47 while lower or no absorption was observed for the other congeners (Stapleton et al., 2004b), which is in accordance with the results of the present study (Table 4.2).

The levels of BDE-47 and BDE-100 found in the brine shrimp (3827 and 467 ng/g lw, respectively; Table 4.1) and zebrafish (5595.8 and 536.2 ng/g lw, respectively; Table 4.2) correlates well with the corresponding concentrations in burbot caught for the present study (14917 and 4572 ng/g lw, respectively; Appendix 1), indicating that those congeners accumulate in zebrafish. However, the concentration of BDE-99 was low in zebrafish (38.3 ng/g lw) compared with brine shrimp (975 ng/g lw) and burbot liver (8890 ng/g lw), suggesting that the zebrafish is capable of metabolizing BDE-99. This is in agreement with Stapleton et al. (2004a; 2004b) reporting a lack of accumulation of BDE-99 in juvenile carp who hypothesised a debromination of BDE-99 to BDE-47 (Stapleton et al., 2004b;Stapleton et al., 2004a). BDE-99 may not accumulate in the zebrafish tissues due to it is metabolized and/or degraded within the gut to other products. The high abundance of BDE-47 in biological extracts may be explained by the ability of some organisms to debrominate higher brominated compounds in the gut, such as BDE-99 (Stapleton et al., 2004a). However, the mechanism is not fully explained, but results from one report suggests that debromination is not catalyzed by CYP (Benedict et al., 2007).

The fact that the concentration of HBCD decreased between brine shrimp (908 ng/g lw; Table 4.1) and zebrafish (279.6 ng/g lw; Table 4.2) in the Mjøsa High exposed group is contradictory to other studies where HBCD has been reported to

bioaccumulate in an aquatic food chain (Morris et al., 2004). This suggests that zebrafish may have a specific ability to metabolize HBCD compared to other aquatic organisms. On the other hand, fish exposed to lower concentrations of the Mjøsa mixture (low and medium refered in the present study) was found with increased HBCD concentration compared to the corresponding brine shrimp, which is in accordance with Morris et al. (2004). The difference in accumulation between the high dose groups compared with the low dose groups is hard to explain. However, some enzymatic mechanisms may differ between the exposure groups leading to the differences in metabolizing this component.

There was also a tendency of some PCB congeners (PCB-28, -99, -101, -110, -128 and -180) to decrease in concentration from brine shrimp to zebrafish in the Mjøsa High group (Table 4.1; Table 4.2). However, the remaining PCBs increased and there was an overall tendency for PCB to slightly biomagnify from brine shrimp (ΣPCB 1200.9 ng/g lw) to zebrafish (ΣPCB 1313.4 ng/g lw).

Lack of accumulation was also observed for DDT and DDD from brine shrimp (177 and 73.1 ng/g lw, respectively; Table 4.1) to zebrafish (40.1 and 41.1 ng/g lw, respectively; Table 4.2), whereas DDE showed an increase (431 ng/g lw in brine shrimp and 536.8 ng/g lw in zebrafish; Mjøsa High exposed group). This is probably explained by the fact that the major breakdown product of DDT is DDE. Biomagnification of DDT has been studied in a Lake Michigan food chain, where total DDT increased 29-fold from plankton to fish [deepwater sculpin (*Myoxocephalus thompsoni*); (Evans et al., 1991)]. In the present study a decrease of total DDT was found, while the DDE itself increased 1.2-fold. Furthermore, the DDE and DDT proportion of total DDT in the zebrafish constituted 63.2 % and 6.5 %, respectively, while it was 67.3 % and 27.8 %, in burbot. The high proportion of DDT found in burbot compared to the low proportion in zebrafish, suggests that there may be ongoing DDT pollution in Lake Mjøsa and/or burbot is a poor metabolizer of DDT.

POP levels found in Losna exposed zebrafish were considerably lower than those measured in Lake Losna burbot. However, a slight increase was found for BDE -47 and HBCD, respectively. Furthermore, the ΣDDT and ΣPCB in Losna exposed zebrafish (81.2 and 233.2 ng/g lw, respectively) were above those found in Mjøsa Low exposed fish (53.1 and 151.1 ng/g lw, respectively).

5.2 Effects on demographic endpoints

In order to assess potential impact of real life mixtures of POPs on the population level, demographic endpoints such as mortality, sex distribution, onset of puberty and growth were measured.

Survival

The survival rate of the experimental animals was considerably higher in the F0 generation than in the F1 generation (70-77 % and 55-67 %, respectively; Fig. 4.2; Fig.4.4). In both generations a marked drop in survival was observed during 9-21 dpf, indicating that this time window was a critical period of development in zebrafish. The present results are in agreement with a previous report on zebrafish exposed to environmental concentrations of EE2 from 0 – 60 dpf, which demonstrated that mortality mainly happened between 5- 22 dpf (Andersen et al., 2003). In the same report survival rates of 47% and 53% were found in EE2 exposed fish and in control fish, respectively. Additionally, Lin and Janz (2006) reported a 77.3 % survival of unexposed zebrafish at 62 dpf.

In the F0 generation there was a significant decrease in survival in Mjøsa exposed fish (Mjøsa High vs. Control; Fig. 4.2). Although, the Mjøsa Low and Mjøsa Medium groups did not differ significantly from Control, there was a dose-related decrease in survival rate with increasing exposure dose (Low, Medium and High). Surprisingly, a significant decrease in survival was also found for Losna exposed fish in the F0 generation. The results from the F0 generation suggested that exposure to natural

mixtures of environmental pollutants affected survival in zebrafish. There is limited data on exposure effects on survival in fish. Zebrafish exposed to environmental binary mixtures of xenoestrogens (nonylphenol and EE2) from 2 to 60 days post hatch (dph) showed a survival between 61 – 88 % depending on the concentration of xenoestrogens (Lin and Janz, 2006), which is in the same range as reported in the present study (70-77 % and 55-67 % for F0 and F1 generations, respectively).

The observed decrease in survival for the Losna and Mjøsa High exposed fish in the F0 generation was carried over to the offspring (F1 generation). In F1 there was a significant decrease in survival during 0-6 dpf (Fig. 4.3), suggesting a transgenerational effect. During the first six days of life the larvae do not eat, and the reduced survival could only be caused by germline transfer. During early embryogenesis in the zebrafish it has been demonstrated that the parental contribution of methylation pattern to the zygote is very similar to that found in mouse (Mhanni and McGowan, 2004). Endocrine disrupting compounds represent potential threats to the imprinting of methylation or acetylation patterns that are transmitted to the next generation (Crews and McLachlan, 2006). Recently it was demonstrated that embryonic exposure to an endocrine disruptor (vinclozolin) had the potential to cause a transgenerational effect on male fertility and testis function, which was transferred to the third generation (Anway et al., 2005). As demonstrated in rodents (Anway et al., 2005;Skinner, 2007), the reduced survival of eggs and larvae of exposed parents during the first six days in the present study, might be a result of POPs affecting the epigenetic programming during embryonic and early larvae development. Data on exposure effects on survival are in general insufficient. However, a study from 1998 conducted on mink fed PCB-contaminated carp, showed that parental exposure to environmental concentrations PCBs had detrimental effects on survival of subsequent generations, litters by chronically exposed parents had greater mortality than controls (Restum et al., 1998).

Although there was a considerable between year differences in survival, the Cox proportional hazard model suggested that there was a significant decrease in survival

across years related to exposure with extracts from Lake Mjøsa and Lake Losna (Table 4.3). The dose level of pollutants acquired in zebrafish (Table 4.2) in the present study is low compared with measured concentrations in wild burbot (Appendix 1), but comparable with POP concentrations found in humans (Smith, 1999;Darnerud et al., 2001;Zhao et al., 2007;Fischer et al., 2006). Further studies focusing more specifically on the critical window of exposure between 6-20 dpf are indicated to investigate what mechanisms are involved in the increased mortality found in exposed groups in this period.

As yet, POP levels have not been measured in the F1 generation. Therefore, at the moment, we regard it irrelevant to speculate whether differences in POP levels between the F0 and F1 generations can have influenced differences in survival rates between the two generations. The lack of reproducibility between the first and second generation could partly be due to differences in exposure scenarios. Exposure of the parent generation was initiated the day they started to eat (6 dpf), whilst their offspring (F1 generation) was exposed during gonadogenesis, embryogenesis as well as under larvae development. Additionally, differences in nutrition between the two generations were indicated by faster growth in F1. The fact that the density of fish and the water level in the zebrafish tanks during 0-14 dpf were higher in F1, may have contributed to lower survival because of longer distance to reach powder feed at the water surface.

Sex ratio

The overall sex distribution for both generations, except for Control-Mjøsa High exposed fish, showed an overweight of males (Table 4.4; Table 4.5). In the F0 generation, Mjøsa exposed fish (Low, Medium and High) taken together and Losna exposed fish, showed a male dominance which differed significantly from Control. In the F1 generation, Mjøsa High-Mjøsa High group showed also significantly more males than the Control-Control. This suggests that mixtures of environmental

pollutants from Lake Losna and Lake Mjøsa may affect the sex distribution of the zebrafish leading to male dominance.

Variable sex ratios have been observed for the zebrafish. A skewed ratio towards females (Orn et al., 2006) as well as skewed sex ratios toward males (Lin and Janz, 2006;Uchida et al., 2002) have been reported in zebrafish. Generally, non-exposed zebrafish have been identified with a varying proportion of males ranging between 33 – 69 % (Orn et al., 2006;Brion et al., 2004). In the present study, male ratios for control fish were 56.6 % and 50 % for F0 and F1 generations, respectively.

A phenotypic assessment of the gender was used to evaluate the sex ratio, which may not be equivalent with genetic sex. The lack of sex chromosomes in zebrafish suggests that sex development may be polygenic or dependent on environmental factors or a combination of both (Devlin and Nagahama, 2002). Another possible explanation for skewed sex ratios in the present study may be a higher early (9-20 dpf) mortality among females exposed to environmental toxins.

The year of study was the main contributor to the variation in sex ratio. Although clear exposure effects were found in the F0 generation, this was not confirmed for comparable groups in the F1 generation. This may be due to the difference in exposure scenarios for these generations, in which the second generation was also exposed during gonadogenesis, embryogenesis and under larvae development. A possible transgenerational effect of Mjøsa and Losna exposure was indicated by the fact that the groups receiving these exposures in F0 maintained a sex ratio skewed towards male dominance, whereas groups exposed to the same mixtures in F1 did not (Table 4.4; Table 4.5). However, this trend was not strong enough to be supported by statistical significance in existing data.

Due to an overall increase in the proportion of males in both generations, one could speculate whether exposure with the Losna and Mjøsa mixtures leads to male dominance in zebrafish. However, the huge difference in sex ratios between years of study and the lack of repeatability in comparable groups across years does not

support such a conclusion. A recent report with zebrafish exposed to environmental binary mixtures of xenoestrogens (nonylphenol and EE2) for 60 days was found to increase the proportions of males (Lin and Janz, 2006). Moreover, an exposure to mixture of environmental concentrations of EE2 (2 ng/L) and the synthetic androgen 17β-trenbolone between 20-60 dpf, resulted in a significantly overweight of males (Orn, 2006). Taken together, it seems likely that mixtures of environmental pollutants that affect the endocrine system, could also affect the sex distribution in zebrafish, and in this case, mixtures harvested from Lake Mjøsa and Lake Mjøsa may leads to male dominance.

Onset of puberty

In both generations the trend was that exposed fish had an earlier onset of puberty than control fish (Table 4.6). Furthermore, an indirect transgenerational effect was indicated in the present study by the fact that exposure in F0 (Losna and Mjøsa High) combined with similar exposure in F1, resulted in an earlier onset of puberty in F1 (Losna-Losna and Mjøsa High-Mjøsa High).

The advanced puberty onset in exposed zebrafish suggests that the Mjøsa and Losna mixtures have potential to interact with the reproductive system by altering sexual maturation. As far as we now, there is little information in the literature on exposure effects on puberty in fish. A transgenerational effect on puberty has been reported in goats where PCB 153 caused a delay the onset of puberty in goat kids exposed *in utero* (Lyche et al., 2004b). Impacts of PCBs on puberty have also been investigated in rats, where an translactational exposure to Aroclor 1254 (32 and 64 µg/g) was found to delay puberty, and impair fertility in female offspring (Sager and Girard, 1994). A delayed onset of sexual maturation has also been shown for guinea pigs after exposure to Clophen A50 (PCB) during gestation (Lundkvist, 1990). Additionally, BDE-99 has also been found to delay onset of puberty in female rat offspring at higher dose levels (Lilienthal et al., 2006).

Body mass and length

The endocrine system is highly sensitive to environmental pollutants. It is not unlikely that changes in hormone levels caused by endocrine disrupting compounds, may lead to underweight or overweight, because growth is partly controlled by hormones. Overall, the mean body mass was 1.3-fold higher (~200 mg increase) in F1 compared with the F0 generation. In general, there was a good agreement between body mass and body length as measures of body size in this study. The increase in body mass from F0 to F1 could partly be due to a higher feeding intensity in F1. The fact that the weight increase was particularly noticeable in the Control-Control group may partly be because spawning was delayed, suggesting that a larger proportion of the energy was used for growth than for egg production in this group.

In the F0 generation the overall mean body mass indicated that all exposure groups had a significantly higher body mass than Control (Fig. 4.6). In contrast, all treatment groups in the F1 generation had a significantly lower body mass compared to Control-Control (Fig. 4.7). Current data and reports in the literature do not provide good explanation for the fact that seemingly similar exposure in two generations can lead to growth stimulation in one generation (F0) and the growth retardation in another (F1). However, a report on zebrafish exposed to environmental binary mixtures of xenoestrogens to 60 dph demonstrated both an increase and a reduction in weight relative to their controls (Lin and Janz, 2006). The binary mixture comprised of EE2 and nonylphenol, of which nonylphenol is a weak ER agonist that can promote and decrease growth rate depending on the concentration of EE2, which is a potent ER agonist. It cannot be excluded that a similar mechanism was involved in the seemingly contradictory between year exposure effects in the present study.

5.3 Effects on gene expression in male zebrafish

Zebrafish exposed for five months to Losna mixture showed no differential gene expression, while in male fish exposed to the Mjøsa High mixture 116 genes were found to be differentially regulated.

Lack of differentially expressed genes after exposure to Losna mixture

The tissue-specific gene expression profiling was not able to detect any differentially expressed genes in neither testis, nor liver tissues, from Losna exposed males compared to the controls (Table 4.9). This could be due to a member of reasons; either technology or biologically related.

DNA microarray technology includes numerous experimental steps as well as a collection of platforms for analyzing the data. In order to determine the reliability of your results all aspects need to be considered. Two extremely important characteristics of performance are accuracy and precision while performing the microarray experiment (van Bakel and Holstege, 2004). Several errors may occur that result in high background levels, consequently leading to illegible slides. Inappropriate washing of slides, dust and mechanical damage on the slides, too high temperature during hybridization as well as leakage of hybridization solution are all critical issues for a successful experiment. The use of external RNA, also known as spike RNA, as controls provides fidelity of the technology in the way that the user knows that only the control RNAs are differentially present and at exactly what amounts (van Bakel and Holstege, 2004). This offers a secured system for determining performance and makes it feasible to discover errors.

However, the slides used in the present study, appeared with normal background levels and control RNA emerged at the expected expression levels, suggesting that technological aspects are not the reason for the lack of detection of differentially regulated genes.

Detecting cellular responses after exposure to low-doses of toxicants may also be complicated by the lack of sensitivity in detecting molecular alterations associated with an adverse effect. It may be that the low dose used in the present study is to low to recover any alterations on gene level in this system. The Losna mixture showed no direct toxicological effects on livers and testis in the zebrafish and a link between gene expression profile and mode of toxicity is therefore impossible to imply.

Taken together, this suggests that there were in fact no differentially expressed genes related to the exposure scenario.

Genes significantly expressed after exposure to Mjøsa High mixture

Based on the results from the gene expression profiling, an attempt was made to link together genes belonging to common signalling pathways. Thus, pathways were identified associated with endocrine regulation, AhR signalling, endoplasmic reticulum stress as well as apoptosis. An overview is outlined in Table 5.1.

Table 5.1: *Cellular processes and the associated differentially expressed genes from liver and testis identified by IPA. Gene name are presented as abbreviations; full name is given in the text or in the list of differentially expressed genes (Section 4.3).*

Process	Liver	Testis
Cell stress	SERP1, SSR1, SEC24D, HSPB8, DUSP1	HSP90AB1, NPM1
Endocrine regulation	COX5A, COX10, MDH2, NCOA4, CALCOCO1	
AhR signalling	RPL7A, RPL10, EDF1, CALCOCO1	HSP90AB1
Apoptosis	ARG2, DUSP1, ACE, JUP, PIM1, HSPB8	HSP90AB1,SMNDC1,PDCD10,TDGF1, C25A5,NPM1

Three pathways were established by IPA among the liver genes from fish exposed to Mjøsa High mixture (Fig. 5.1 – 5.3).

Cell stress

Among the differentially expressed genes in liver tissue from male zebrafish after five months of exposure, were genes associated to a stress response in the endoplasmic reticulum. These genes included: stress-associated endoplasmic reticulum protein (SERP1), signal-sequence receptor α (SSR1), and Sec24 related protein D (SEC24D). IPA associated these genes with a common transcription regulator, XPB1 (Fig. 5.1).

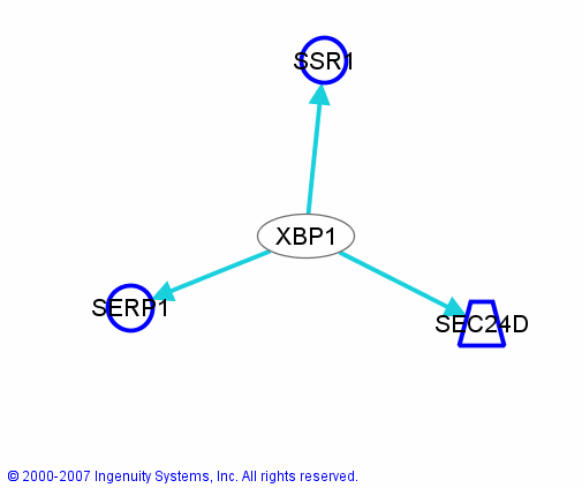


Figure 5.1: *Endoplasmic reticulum-related genes (SERP1, SSR1, SEC24D) regulated by a common transcription factor, XBP1, as identified in IPA. Differentially expressed genes are displayed in dark blue; nodes added by IPA are displayed in grey. Interactions between nodes are identified by light blue arrows.*

These genes (SERP1, SSR1, SEC24D) appeared downregulated and were nicely connected to the transcription factor XBP1, which is involved in the unfolded protein response (UPR) (Kohno, 2007). The UPR is a stress response associated with endoplasmic reticulum when cells suffer from accumulation of unfolded or misfolded proteins (Kohno, 2007). A recent study has demonstrated that SERP1 knockout mice showed a postnatal growth retardation and increased mortality (Hori et al., 2006). The finding that XBP1 has been identified in zebrafish (Hu et al., 2007), together with the down regulation of genes involved in the UPR might reflect the higher mortality seen

in the Mjøsa High exposed groups. The high mortality in the Mjøsa High exposed groups may be supported by the induced stress response.

Furthermore, heat shock protein 22 (HSPB8) as well as dual specificity phosphatase 1 (DUSP1), also involved in stress response (IPA, 2007), were also found to be affected in the liver cells of the zebrafish. HSPB8 were downregulated and DUSP1 appeared upregulated.

In the testis , another heat shock protein (HSP90AB), also involved in cellular stress response, was found to be upregulated (IPA, 2007). These findings suggest that the cells in the zebrafish exposed to environmental pollutants react to the toxic challenge with a cellular stress response.

As the exposure doses used in the present study are also relevant in a human context, this suggests the potential of these compounds to induce a similar stress response. However, one should bear in mind that these genes are just small pieces in a huge network of connected genes.

Apoptosis

Prolonged cell stress, such as oxidative stress are known to result in the activation of apoptotic machinery (Verhaegen et al., 1995). The threshold for the induction of apoptosis is set through a number of pro or antiapoptotic genes. IPA identified a cluster of differentially expressed genes in the liver of the Mjøsa High exposed fish associated with a key enzyme involved in the execution of the apoptotic machinery, caspase 3 (CASP3; Fig. 5.2).

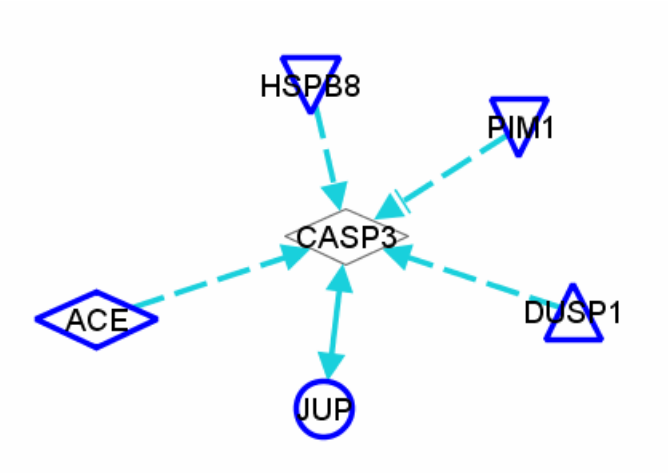


Figure 5.2: *Differentially expressed genes (ACE, HSPB8, PIM1, DUSP1, JUP) associated with the apoptosis related gene caspase 3 (CASP3). Differentially expressed genes are displayed in dark blue; nodes added by IPA are displayed in grey. Interactions between nodes are identified by light blue arrows. Dashed arrows represent indirect interaction and solid arrows represent direct interactions.*

The genes associated with apoptotic signalling includes HSPB8, dual specificity phosphatise 1 (DUSP1), the proto-oncogene 1 (PIM1), angiotensin converting enzyme (ACE), and junction plakoglobin (JUP; Fig. 5.2). These are closely associated with CASP3, a key enzyme in apoptosis (IPA, 2007). All was identified to be able to regulate activity of CASP3. ACE can modulate the subcellular localization of CASP3, and cleavage of JUP can be increased by CASP3 (IPA, 2007).

In addition, expression of the arginase type 2 (ARG2) gene was found to be affected. This gene is known to regulate production of spermine and spermidine (IPA, 2007). These polyamines are known inhibitors of apoptosis (Brune et al., 1991).

Similarly in the testis, regulators of apoptosis were found to be affected. These include HSP90AB1, survival motor neuron domain containing 1 (SMNDC1), programmed cell death 10 (PDCD10), teratocarcinoma-derived growth factor 1 (TDGF1), solute carrier family 25 member 5 (SLC25A5) and nucleophosmin 1 (NPM1).

Taken together, this indicates that exposure to environmental pollutants can affect the expression of genes setting the cellular balance between life and death.

Endocrine regulation

In the liver, genes encoding for proteins located in the mitochondria, the main organelle associated with steroid production, were found to be affected. These included cytochrome c oxidase subunit 5α (COX5A) and the related member cytochrome c oxidase assembly protein 10 (COX10). In addition, expression of malate dehydrogenase 2 (MDH2) gene was found to be affected (Fig. 5.3).

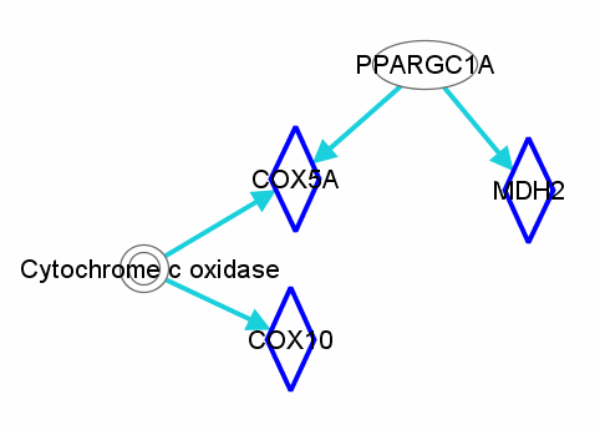


Figure 5.3: *Differentially expressed mitochondrial function-related genes (COX10, COX5A, MDH2) and their relation as identified in IPA. Differentially expressed genes are displayed in dark blue; nodes added by IPA are displayed in grey. Interactions between nodes are identified by light blue arrows.*

IPA identified a common transcriptional coactivator, PPAGC1A (peroxisome proliferator-activated receptor γ, coactivator 1 α), capable of regulating the expression of both COX5A and MDH2. PPAGC1A has been identified to be involved in oestrogen receptor signalling as well as xenobiotic metabolism signalling (IPA, 2007).

In addition, genes involved in steroid signalling were differentially regulated. For example, nuclear receptor coactivator 4 (NCOA4), which is known to be involved in regulation of both oestrogen receptor and androgen receptor (IPA, 2007). Another affected gene associated with the steroid hormone receptor signalling was the calcium binding and coiled-coil domain 1 (CALCOCO1).

This suggests that the Mjøsa High dose may have an impact on the steroidogenesis and steroid signalling in the zebrafish.

Apparently, not many differentially expressed genes associated to an endocrine effect were detected in liver or testis tissue. One affected gene was the vitellogenin 1; however, this gene was downregulated suggesting that the Mjøsa High mixture do not have an estrogenic effect. Additionally, hydroxysteroid dehydrogenase like 2 (HSDL2) was found downregulated, where its protein product is the main enzyme responsible for inactivating oestrogen and androgens (IPA, 2007). However, this may have had an impact on the steroidogensis in the Mjøsa High exposed zebrafish.

AhR signalling

CALCOCO1 is also found to regulate AhR as well as ARNT (IPA, 2007), indicating an association with the AhR pathway. An additional gene, HSP90AB, is also involved in the AhR pathway, was found to be upregulated. The protein product of HSP90AB; HSP90, is a chaperone of the AhR and preventing it to enter the cell nucleus before a ligand binds. An upregulation of HSP90AB suggest an inhibitory effect on the receptor signalling. HSP90 is also a mediator of crosstalk between the AhR and ER signal transduction pathways (Safe, 2001).

5.4 Critical windows of exposure

In the present study there was a marked drop in survival during 9-20 dpf for both generations (Fig. 4.2; Fig. 4.4); suggesting that this period may be a critical window of exposure for the zebrafish. The pattern of survival by time was observed for all

groups, including control fish, suggesting that factors other than exposure by pollutants are important for survival in this period.

During the first three weeks in life, zebrafish develop undifferentiated ovary-like gonads regardless of gender (Uchida et al., 2002). After this stage the oocytes in the females grow to maturation, while in males the oocytes degenerate and spermatocytes develop along with growth of the testes. This sex differentiation is a male specific event caused by apoptosis (Uchida et al., 2002). However, this period is most likely critical to exposure of pollutants with a potential to interfere with the endocrine system. Suppression of apoptosis has been demonstrated *in vitro* with mature follicles from rainbow trout *(Oncorhynchus mykiss)* exposed to 17β-estradiol (Janz and Van der, 1997), suggesting that this period may also be sensitive for the sex distribution of the zebrafish.

A study conducted on zebrafish exposed to 10 ng/L of EE2 during three stages of gonadal differentiation; juvenile hermaphroditic stage when all gonads are immature like ovaries (15 - 42 dpf), gonad transition stage when the hermaphroditic ovaries develop into either testis or mature ovaries (43 - 71 dpf), and the premature stage of testis and ovaries (72 - 99 dpf), showed that only exposure during 43 – 71 dpf when the transition of gonads took place, induced a delay in the onset of spawning and reduced fertilization success of adult fish (Maack and Segner, 2004). The result from this experiment suggests that other life stages are also important when it comes to defining critical windows of exposure.

The gene expression profiling identified differentially expressed genes associated to apoptosis in the Mjøsa High exposed group (Fig. 5.2). Although these genes were detected in the adult life of the zebrafish, one should not exclude the possibility that POPs have influenced the apoptotic machinery in early life stages, supporting that this time window may be critical for survival. Furthermore, genes associated to cell stress in the endoplasmic reticulum was also detected to be differentially expressed, suggesting that an induced stress response also may have contributed to the decrease in survival during this time period.

We have speculated whether the size of the mouth opening of the zebrafish larvae is vital for the survival. If it is too small the ingestion of food will be impaired and the survival probability decreased. It is not known whether the exposure effects observed in the present study were related to the ability to ingest food. Little information is available on survival in teleost fish in general. Further studies into the critical window of exposure during 9-21 dpf is therefore needed to assess exposure effects on survival in more detail.

5.5 Biomarkers as potential indicators of mixture effects

In order to assess the impact of environmental mixture effects in the zebrafish model, EROD and vitellogenin was analyzed.

EROD

Exposures to real life mixtures of POPs from Lake Losna and Lake Mjøsa resulted in small variations in the induction of EROD activity in the gill arches of the zebrafish. Nevertheless, the control group differed significantly from the other treatment groups in which EROD activity was slightly increased. Compared with Control a 1.3-fold increase was found in Mjøsa Low, which had a higher EROD activity than all other groups.

The low induction of EROD activity seen in the experimental animals might be explained by the mixtures may contain low levels of dioxin or dioxin-like compounds, which has not been measured in the present study. However, a NIVA report from 2001 states dioxin levels (ΣPCDD/PCDF) to be 178 ng/kg ww in burbot liver from Lake Mjøsa (Fjeld et al., 2001), suggesting that most likely these mixtures also contain dioxin or dioxin-like compounds. Zebrafish exposed to 0.75 µg/g of TCDD for three days, showed an 17-fold increase in EROD induction relative to their controls (Troxel et al., 1997). Taken together, this suggests that most likely an induction in EROD activity in the zebrafish would have been expected.

The basal gill EROD activity observed in the present study was somewhat higher than observed for other fish species, such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), Arctic charr (*Salvelinus alpinus*), Atlantic cod (*Gadus morhua*) and saithe (*Pollachius virens*) (Jonsson et al., 2002;Jonsson et al., 2003). This finding, together with an overall small variation in EROD induction, suggests that the used EROD gill filament assay did not reveal a strong CYP1A induction caused by exposure in zebrafish. Previous gill filament experiments (Jonsson et al., 2003;Jonsson et al., 2002) were performed with water exposure to which a gill assay might prove to be more sensitive.

The potential of a compound to induce levels of biotransformation enzymes is dependent on its ability to bind to AhR. The structural similarity of certain PBDE congeners has led to investigation of the ability of these compounds to act as agonists to AhR and subsequently inducing CYP1A. However, an inhibition of EROD activity when co-exposed with TCDD has been demonstrated for a variety of PCB congeners (Besselink et al., 1998). *In vitro* studies with human and rat hepatoma cell lines exposed to environmentally relevant mixtures of PBDEs (BDE-47, -77, -99, -100, -153, -154, -183, -209) showed no agonist potential, but rather an antagonistic or inhibitory effect when these cells were co-exposed to PBDEs together with TCDD (Peters et al., 2004). Furthermore, a similar effect between PBDEs and TCDD has also been demonstrated in primary carp hepatocytes, where individual PBDE congeners (BDE-47, -99 and -153) was found to significantly reduce the TCDD-induced EROD activity (Kuiper et al., 2004). In the same study, it was also recognized a dose dependent decrease in EROD activity with increasing dose levels of the BDE congeners. After long term exposure to a mixture of PCBs, Örn et al. (1998) found also decreased activity. After 4 weeks of exposure the mixture had the potential to elevate EROD levels in hepatic tissue in zebrafish (Orn et al., 1998). However, it was further reported that after 13 weeks of exposure, the highest PCB mixture reduced the EROD activity. This was likely due to some adaptive response or intracellular damage of the hepatocytes.

Another compound which has been associated with the ability to inhibit EROD activity is HBCD (Ronisz et al., 2004). In this study, where rainbow trout was injected with HBCD for 28 days, it was demonstrated that a 500 mg/kg dose led to an inhibition of EROD activity. Furthermore, long-term exposure of European flounder (*Platichtys flesus*) to environmentally relevant concentrations of HBCD was found with a low EROD induction (Kuiper et al., 2007). This suggests that HBCD might have contributed to the low EROD activity detected in the present study.

The apparent lack of CYP1A induction in the zebrafish might partly be associated with a developed resistance to these chemicals. Wild fish populations have been suggested to build up a resistance when living in chronically exposed environment (Nacci et al., 1999;Brammell et al., 2004). Nacci et al. (1999) stated that fish constantly exposed to dioxin-like PCBs showed the same levels of CYP1A as fish from a reference population. In addition, it was shown that the observed resistance was inherited and suggested that the long term effect of dioxin-like compounds included a genetic restructuring of the wild fish population. Furthermore, it was thought that the molecular mechanism associated with resistance involved the AhR signalling. Thus, the mixtures used in the present study which consist of high levels of PBDEs as well as PCBs, suggests that a similar antagonistic mechanism may have occurred in the zebrafish after long term exposure to environmentally relevant concentrations of the mixtures.

Additionally, EROD activity in scup (*Stenotomus chrysops*) by PCB-126 was found not to be induced in liver by a low dose of PCB while a high dose suppressed the protein and activity (Schlezinger and Stegeman, 2001). A similar phenomenon was seen in the present study, where the highest Mjøsa dose was found to show the lowest induced EROD activity (0.0592 pmol/resorufin/gill arch/min) whilst the lowest Mjøsa dose induced most (0.0684 pmol/resorufin/gill arch/min). However, the Losna mixture was also found to induce in the same amount (0.0595 pmol/resorufin/gill arch/min) as Mjøsa High.

Based on what is written above, it cannot be excluded that the EROD gill filament assay has a low sensitivity for detecting dioxin or dioxin-like compounds, and subsequently measure EROD activity in the zebrafish exposed to real life mixtures of POPs.

Vitellogenin

An eleven-fold increase in vitellogenin levels was detected for the Mjøsa Low exposed fish compared to control fish. This stands in contrast to the other treatment groups which showed merely a 1.6-, 2.1-and 3.3-fold elevation in Losna, Mjøsa Medium and Mjøsa High exposed fish, respectively. However, the mean vitellogenin concentrations in control zebrafish was 9985 ng/g, which is higher than previously reported; 500 ng/g (Orn et al., 2006) and 70 ng/g (Orn et al., 2003) for non-exposed male fish. One suggestion for the high Vtg levels in controls in the present experiment, is the chronically exposure to low levels of estrogenic compounds through the system water, which may have triggered the vitellogenic response.

The potency of environmental oestrogens to induce vitellogenin levels differ. When appearing in mixtures some chemicals may strengthen or antagonize the Vtg production. A mixture of a variety of oestrogenic compounds was found to show a more pronounced vitellogenin response than a single oestrogenic chemical at the same concentration (Sumpter and Jobling, 1995). Nonylphenol (a derivative of surfactants) is found to act both additively and non-additively in a combination with a potent ER agonist, depending on the dose of the chemicals (Lin and Janz, 2006). Furthermore, *o-p'*-DDT, Aroclor 1254 and γ-HCH was found to only slightly or not induce Vtg levels in Atlantic salmon (Arukwe et al., 2000). When these chemicals were administered in a combination with nonylphenol, the Vtg levels was elevated.

Thus, it might be that the different POPs in Mjøsa Low exposed fish behaved in an additive manner, whilst higher doses antagonized the Vtg induction like in Mjøsa Medium and Mjøsa High groups. However, the weak increase of Vtg in Mjøsa High

exposed fish is related to the downregulated vitellogenin gene observed in the livers of male fish treated with Mjøsa High mixture. The high background levels as well as the vague induction of Vtg in general, suggest that the mixtures did not have a strong estrogenic effect in zebrafish.

Several POPs, such as TCDD which as yet has not been measured in the mixtures, could explain the low Vtg induction observed. TCDD has been found to adversely impact spawning success and decrease egg production as well as decrease serum vitellogenin concentrations in zebrafish (Heiden et al., 2006).

6. Main conclusions

In this thesis the principal objective was to study effects of real life mixtures of brominated flame retardants and organochlorine chemicals harvested from burbot in Lake Losna and Lake Mjøsa in the zebrafish model. The concentration ranges of POPs measured in zebrafish were lower than in burbot originating from Lake Mjøsa, but comparable to concentrations previously reported in humans and wildlife. Results indicated that mixtures originating from Mjøsa as well as Losna had the potential to cause harmful effects in zebrafish.

On the population level exposure to Mjøsa and Losna mixtures caused a decrease in survival in both generations. The marked drop in survival during 9-20 dpf suggested that this period may be a critical window of development in the zebrafish.

In the parent (F0) generation there was a higher proportion of males and an earlier onset of puberty in Losna and Mjøsa exposed groups. Exposure with Losna and Mjøsa mixtures increased body mass and length in the parent generation (F0), while in the next generation (F1) the same exposures were associated with a decrease in body mass and length.

An indirect transgenerational effect of onset of puberty was suggested by the fact that exposure in F0 (Losna and Mjøsa High) combined with similar exposure in F1 resulted in an earlier onset of puberty in F1 (Losna-Losna and Mjøsa High-Mjøsa High). In terms of sex ratio, a possible transgenerational effect of Mjøsa and Losna exposure was also indicated by the fact that the groups receiving these exposures in F0 maintained a sex ratio skewed towards male dominance, whereas groups exposed to the same mixtures in F1 did not.

There were no genome-wide effects after five months of exposure to the Losna mixture, while exposure to the Mjøsa High mixture affected expression of genes linked to cellular stress, as well as genes associated with the apoptotic machinery.

The biomarkers used in the present study (EROD; Vtg) did not reveal strong estrogenic effects or dioxin-like effects that could be associated with exposure with real life mixtures of POPs.

In conclusion, exposures with real life mixtures of POPs harvested from Lake Mjøsa and Lake Losna in the zebrafish model were associated with significant effects, both at the population level and at the genomic level. The dominating effects were related to reproduction, development and survival. There was stronger evidence in the data for exposure effects linked to the higher polluted location, Lake Mjøsa, compared with the lower polluted location, Lake Losna.

7. Future perspectives

The lack of reproducibility between the F0 and F1 generation raises the question of how robust the zebrafish model is for studying mixture effects at the population level. However, the present results clearly indicated exposure effects at very low concentration levels.

Further investigations into the effects linked to exposures with natural mixtures of POPs should be focused on shorter time windows relevant for reproductive and developmental endpoints. Of special interest in this context would be time windows linked to the first month after fertilization.

The present data indicated that exposure effects can be transferred to the next generation. The zebrafish model is well suited for studying mechanisms of transgenerational effects, since embryos are easily accessible. Additionally, embryonic stem cells with different life history can be used for more detailed studies of effects linked to differentiation and development.

The use of gene expression profiling in the present study has provided a possibility to identify novel biomarkers, which may prove to be more sensitive for detection of biological responses, in order to monitor aquatic pollution and study the impact of chemicals or toxins on marine and freshwater populations. Gene expression profiling, proteomics and other technologies, including also epigenetic studies will be used in future studies in order to get a better picture of exposure effects of POPs and their mechanisms of action.

References

1. Alestrom,P., Holter,J.L., and Nourizadeh-Lillabadi,R. (2006). Zebrafish in functional genomics and aquatic biomedicine. Trends Biotechnol 24:15-21.

2. Andersen,A., Kasperlik-Zaluska,A.A., and Warren,D.J. (1999). Determination of mitotane (o,p-DDD) and its metabolites o,p-DDA and o,p-DDE in plasma by high-performance liquid chromatography. Ther Drug Monit 21:355-359.

3. Andersen,L., Holbech,H., Gessbo,A., Norrgren,L., and Petersen,G.I. (2003). Effects of exposure to 17alpha-ethinylestradiol during early development on sexual differentiation and induction of vitellogenin in zebrafish (Danio rerio). Comp Biochem Physiol C Toxicol Pharmacol 134:365-374.

4. Andersson,O. and Blomkvist,G. (1981). Polybrominated aromatic pollutants in fish in Sweden. Chemosphere 10:347-350.

5. Anway,M.D., Cupp,A.S., Uzumcu,M., and Skinner,M.K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308:1466-1469.

6. Arukwe,A., Celius,T., Walther,B.T., and Goksoyr,A. (2000). Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (Salmo salar). Aquat Toxicol 49:159-170.

7. ATSDR. Chlordane. CAS # 57-74-9. 1995. US Dept of Health & Human Services, Agency for Toxic Substances and Disease Registry. Division of Toxicology ToxFAQs™.
   Ref Type: Report

8. ATSDR. Hexachlorobenzene. CAS # 118-74-1. 2002. US Dept of Health & Human Services, Agency for Toxic Substances and Disease Registry. Division of Toxicology ToxFAQs™.
   Ref Type: Report

9. Baars,A.J., Bakker,M.I., Baumann,R.A., Boon,P.E., Freijer,J.I., Hoogenboom,L.A., Hoogerbrugge,R., van Klaveren,J.D., Liem,A.K., Traag,W.A., and de,V.J. (2004). Dioxins, dioxin-like PCBs and non-dioxin-like PCBs in foodstuffs: occurrence and dietary intake in The Netherlands. Toxicol Lett 151:51-61.

10. Benedict,R.T., Stapleton,H.M., Letcher,R.J., and Mitchelmore,C.L. (2007). Debromination of polybrominated diphenyl ether-99 (BDE-99) in carp (Cyprinus carpio) microflora and microsomes. Chemosphere 69:987-993.

11. Besselink,H.T., Denison,M.S., Hahn,M.E., Karchner,S.I., Vethaak,A.D., Koeman,J.H., and Brouwer,A. (1998). Low inducibility of CYP1A activity by

polychlorinated biphenyls (PCBs) in flounder (Platichthys flesus): characterization of the Ah receptor and the role of CYP1A inhibition. Toxicol Sci 43:161-171.

12. Birnbaum,L.S. (1994). Endocrine effects of prenatal exposure to PCBs, dioxins, and other xenobiotics: implications for policy and future research. Environ Health Perspect 102:676-679.

13. Birnbaum,L.S. and Staskal,D.F. (2004). Brominated flame retardants: cause for concern? Environ Health Perspect 112:9-17.

14. Bonefeld-Jorgensen,E.C., Andersen,H.R., Rasmussen,T.H., and Vinggaard,A.M. (2001). Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. Toxicology 158:141-153.

15. Bornman,M.S., Pretorius,E., Marx,J., Smit,E., and van der Merwe,C.F. (2007). Ultrastructural effects of DDT, DDD, and DDE on neural cells of the chicken embryo model. Environ Toxicol 22:328-336.

16. Brammell,B.F., Price,D.J., Birge,W.J., and Elskus,A.A. (2004). Apparent lack of CYP1A response to high PCB body burdens in fish from a chronically contaminated PCB site. Mar Environ Res 58:251-255.

17. Brevik,E.M. (1978). Gas chromatograhic method for the determination of organochlorine pesticides in human milk. Bull Environ Contam Toxicol 19:281-286.

18. Brion,F., Tyler,C.R., Palazzi,X., Laillet,B., Porcher,J.M., Garric,J., and Flammarion,P. (2004). Impacts of 17beta-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (Danio rerio). Aquat Toxicol 68:193-217.

19. Brown,F.R., Winkler,J., Visita,P., Dhaliwal,J., and Petreas,M. (2006). Levels of PBDEs, PCDDs, PCDFs, and coplanar PCBs in edible fish from California coastal waters. Chemosphere 64:276-286.

20. Brune,B., Hartzell,P., Nicotera,P., and Orrenius,S. (1991). Spermine prevents endonuclease activation and apoptosis in thymocytes. Exp Cell Res 195:323-329.

21. Buser,H.R. (1986). Polybrominated Dibenzofurans and Dibenzo-p-dioxins: Thermal Reaction Products of polybrominated Diphenyl Ether Flame Retardants. Environ Sci Technol 20:404-408.

22. California State Senate. California State Senate, Bill # AB 302. 2003.
Ref Type: Report

23. Casarett,L.J. and Doull,J. (2001). Toxicology: The basic science of poisons. Kansas City, Kansas: The McGraw-Hill Companies, Inc.

24. Chauhan,K.R., Kodavanti,P.R., and McKinney,J.D. (2000). Assessing the role of ortho-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. Toxicol Appl Pharmacol 162:10-21.

25. Chen,C.W., Hurd,C., Vorojeikina,D.P., Arnold,S.F., and Notides,A.C. (1997). Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells. Biochem Pharmacol 53:1161-1172.

26. Cocco,P., Blair,A., Congia,P., Saba,G., Ecca,A.R., and Palmas,C. (1997). Long-term health effects of the occupational exposure to DDT. A preliminary report. Ann N Y Acad Sci 837:246-256.

27. Colborn,T., vom Saal,F.S., and Soto,A.M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378-384.

28. Collett,D. (2001). Modelling survival data in medical research. Boca Raton: Chapman and Hall.

29. Corsolini,S., Kannan,K., Imagawa,T., Focardi,S., and Giesy,J.P. (2002). Polychloronaphthalenes and other dioxin-like compounds in Arctic and Antarctic marine food webs. Environ Sci Technol 36:3490-3496.

30. Corsolini,S., Sara,G., Borghesi,N., and Focardi,S. (2007). HCB, p,p'-DDE and PCB ontogenetic transfer and magnification in bluefin tuna (Thunnus thynnus) from the Mediterranean Sea. Environ Sci Technol 41:4227-4233.

31. Crews,D. and McLachlan,J.A. (2006). Epigenetics, evolution, endocrine disruption, health, and disease. Endocrinology 147:S4-10.

32. Dahm,R. and Geisler,R. (2006). Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol (NY) 8:329-345.

33. Darnerud,P.O. (2003). Toxic effects of brominated flame retardants in man and in wildlife. Environ Int 29:841-853.

34. Darnerud,P.O., Aune,M., Larsson,L., and Hallgren,S. (2007). Plasma PBDE and thyroxine levels in rats exposed to Bromkal or BDE-47. Chemosphere 67:S386-S392.

35. Darnerud,P.O., Eriksen,G.S., Johannesson,T., Larsen,P.B., and Viluksela,M. (2001). Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. Environ Health Perspect 109 Suppl 1:49-68.

36. de Wit,C.A. (2002). An overview of brominated flame retardants in the environment. Chemosphere 46:583-624.

37. Devlin,R.H. and Nagahama,Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208:191-364.

38. Dewailly,E., Ayotte,P., Bruneau,S., Gingras,S., Belles-Isles,M., and Roy,R. (2000). Susceptibility to infections and immune status in Inuit infants exposed to organochlorines. Environ Health Perspect 108:205-211.

39. Edmunds,J.S., McCarthy,R.A., and Ramsdell,J.S. (2000). Permanent and functional male-to-female sex reversal in d-rR strain medaka (Oryzias latipes) following egg microinjection of o,p'-DDT. Environ Health Perspect 108:219-224.

40. Eriksson,P., Fischer,C., Wallin,M., Jakobsson,E., and Fredriksson,A. (2006). Impaired behaviour, learning and memory, in adult mice neonatally exposed to hexabromocyclododecane (HBCDD). Environemental Toxicology and Pharmacology 21:317-322.

41. Eriksson,P., Viberg,H., Jakobsson,E., Orn,U., and Fredriksson,A. (2002). A brominated flame retardant, 2,2',4,4',5-pentabromodiphenyl ether: uptake, retention, and induction of neurobehavioral alterations in mice during a critical phase of neonatal brain development. Toxicol Sci 67:98-103.

42. Evans,M.S., Noguchi,G.E., and Rice,C.P. (1991). The biomagnification of polychlorinated biphenyls, toxaphene, and DDT compounds in a Lake Michigan offshore food web. Arch Environ Contam Toxicol 20:87-93.

43. Fischer,D., Hooper,K., Athanasiadou,M., Athanassiadis,I., and Bergman,A. (2006). Children show highest levels of polybrominated diphenyl ethers in a California family of four: a case study. Environ Health Perspect 114:1581-1584.

44. Fjeld, E., Knutzen, J., Brevik, E. M., Schlabach, M., Skotvold, T., Borgen, A. R., and Wiborg, M. L. Halogenerte organiske miljøgifter og kvikksølv i norsk ferskvannsfisk, 1995-1999. Rapport 827/01. 2001.
Ref Type: Report

45. Fjeld, E., Schlabach, M., Berge, J. A., Eggen, T., Snilsberg, P., Kãllberg, G., Rognerud, S., Enge, E. K., Borgen, A., and Gundersen, H. Kartlegging av utvalge nye organiske miljøgifter - bromerte flammehemmere, klorerte parfiner, bisfenol A og triclosan. TA-2006/2004. 2004.
Ref Type: Report

46. Goksoyr,A. (2006). Endocrine disruptors in the marine environment: mechanisms of toxicity and their influence on reproductive processes in fish. J Toxicol Environ Health A 69:175-184.

47. Grinwis,G.C., Vethaak,A.D., Wester,P.W., and Vos,J.G. (2000). Toxicology of environmental chemicals in the flounder (Platichthys flesus) with emphasis on the immune system: field, semi-field (mesocosm) and laboratory studies. Toxicol Lett 112-113:289-301.

48. Hale,R.C., Alaee,M., Manchester-Neesvig,J.B., Stapleton,H.M., and Ikonomou,M.G. (2003). Polybrominated diphenyl ether flame retardants in the North American environment. Environ Int 29:771-779.

49. Hale,R.C., La Guardia,M.J., Harvey,E.P., Mainor,T.M., Duff,W.H., and Gaylor,M.O. (2001). Polybrominated diphenyl ether flame retardants in Virginia freshwater fishes (USA). Environ Sci Technol 35:4585-4591.

50. Hallgren,S. and Darnerud,P.O. (2002). Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats-testing interactions and mechanisms for thyroid hormone effects. Toxicology 177:227-243.

51. Hansen,L.G. (1998). Stepping backward to improve assessment of PCB congener toxicities. Environ Health Perspect 106 Suppl 1:171-189.

52. Hany,J., Lilienthal,H., Sarasin,A., Roth-Harer,A., Fastabend,A., Dunemann,L., Lichtensteiger,W., and Winneke,G. (1999). Developmental exposure of rats to a reconstituted PCB mixture or aroclor 1254: effects on organ weights, aromatase activity, sex hormone levels, and sweet preference behavior. Toxicol Appl Pharmacol 158:231-243.

53. Hartmann,P.C., Burkhardt-Holm,P., and Giger,W. (2007). Occurrence of polybrominated diphenyl ethers (PBDEs) in brown trout bile and liver from Swiss rivers. Environ Pollut 146:107-113.

54. Heiden,T.K., Carvan,M.J., III, and Hutz,R.J. (2006). Inhibition of follicular development, vitellogenesis, and serum 17beta-estradiol concentrations in zebrafish following chronic, sublethal dietary exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Sci 90:490-499.

55. Heiden,T.K., Hutz,R.J., and Carvan,M.J., III (2005). Accumulation, tissue distribution, and maternal transfer of dietary 2,3,7,8,-tetrachlorodibenzo-p-dioxin: impacts on reproductive success of zebrafish. Toxicol Sci 87:497-507.

56. Heimler,I., Rawlins,R.G., Owen,H., and Hutz,R.J. (1998). Dioxin perturbs, in a dose- and time-dependent fashion, steroid secretion, and induces apoptosis of human luteinized granulosa cells. Endocrinology 139:4373-4379.

57. Hickey,J.P., Batterman,S.A., and Chernyak,S.M. (2006). Trends of chlorinated organic contaminants in great lakes trout and walleye from 1970 to 1998. Arch Environ Contam Toxicol 50:97-110.

58. Hill,A., Howard,C.V., Strahle,U., and Cossins,A. (2003). Neurodevelopmental defects in zebrafish (Danio rerio) at environmentally relevant dioxin (TCDD) concentrations. Toxicol Sci 76:392-399.

59. Holladay,S.D. (1999). Prenatal immunotoxicant exposure and postnatal autoimmune disease. Environ Health Perspect 107 Suppl 5:687-691.

60. Hooper,K. and McDonald,T.A. (2000). The PBDEs: an emerging environmental challenge and another reason for breast-milk monitoring programs. Environ Health Perspect 108:387-392.

61. Hori,O., Miyazaki,M., Tamatani,T., Ozawa,K., Takano,K., Okabe,M., Ikawa,M., Hartmann,E., Mai,P., Stern,D.M., Kitao,Y., and Ogawa,S. (2006). Deletion of SERP1/RAMP4, a component of the endoplasmic reticulum (ER) translocation sites, leads to ER stress. Mol Cell Biol 26:4257-4267.

62. Hruska,R.E. and Olson,J.R. (1989). Species differences in estrogen receptors and in the response to 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure. Toxicol Lett 48:289-299.

63. Hu,M.C., Gong,H.Y., Lin,G.H., Hu,S.Y., Chen,M.H., Huang,S.J., Liao,C.F., and Wu,J.L. (2007). XBP-1, a key regulator of unfolded protein response, activates transcription of IGF1 and Akt phosphorylation in zebrafish embryonic cell line. Biochem Biophys Res Commun 359:778-783.

64. Ikonomou,M.G., Rayne,S., and Addison,R.F. (2002). Exponential increases of the brominated flame retardants, polybrominated diphenyl ethers, in the Canadian Arctic from 1981 to 2000. Environ Sci Technol 36:1886-1892.

65. Ingelido,A.M., Ballard,T., Dellatte,E., di,D.A., Ferri,F., Fulgenzi,A.R., Herrmann,T., Iacovella,N., Miniero,R., Papke,O., Porpora,M.G., and De,F.E. (2007). Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in milk from Italian women living in Rome and Venice. Chemosphere 67:S301-S306.

66. IPA. Ingenuity Pathways Analysis. http://www.ingenuity.com/index.html . 2007. Ref Type: Internet Communication

67. Janz,D.M. and Van der,K.G. (1997). Suppression of apoptosis by gonadotropin, 17beta-estradiol, and epidermal growth factor in rainbow trout preovulatory ovarian follicles. Gen Comp Endocrinol 105:186-193.

68. Jaraczewska,K., Lulek,J., Covaci,A., Voorspoels,S., Kaluba-Skotarczak,A., Drews,K., and Schepens,P. (2006). Distribution of polychlorinated biphenyls, organochlorine pesticides and polybrominated diphenyl ethers in human umbilical cord serum, maternal serum and milk from Wielkopolska region, Poland. Sci Total Environ 372:20-31.

69. Jonsson,E.M., Abrahamson,A., Brunstrom,B., and Brandt,I. (2006). Cytochrome P4501A induction in rainbow trout gills and liver following exposure to waterborne indigo, benzo[a]pyrene and 3,3',4,4',5-pentachlorobiphenyl. Aquat Toxicol 79:226-232.

70. Jonsson,E.M., Brandt,I., and Brunstrom,B. (2002). Gill filament-based EROD assay for monitoring waterborne dioxin-like pollutants in fish. Environ Sci Technol 36:3340-3344.

71. Jonsson,M., Abrahamson,A., Brunstrom,B., Brandt,I., Ingebrigtsen,K., and Jorgensen,E.H. (2003). EROD activity in gill filaments of anadromous and marine fish as a biomarker of dioxin-like pollutants. Comp Biochem Physiol C Toxicol Pharmacol 136:235-243.

72. Kaphalia,B.S., Takroo,R., Mehrotra,S., Nigam,U., and Seth,T.D. (1990). Organochlorine pesticide residues in different Indian cereals, pulses, spices, vegetables, fruits, milk, butter, Deshi ghee, and edible oils. J Assoc Off Anal Chem 73:509-512.

73. Kato,Y., Haraguchi,K., Shibahara,T., Yumoto,S., Masuda,Y., and Kimura,R. (1999). Reduction of thyroid hormone levels by methylsulfonyl metabolites of tetra- and pentachlorinated biphenyls in male Sprague-Dawley rats. Toxicol Sci 48:51-54.

74. Kelce,W.R., Stone,C.R., Laws,S.C., Gray,L.E., Kemppainen,J.A., and Wilson,E.M. (1995). Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. Nature 375:581-585.

75. KEMI. KEMI Reports. Hexabromocyclododecan (HBCD) and tetrabromobisphenol A (TBBPA). 3/06 Summary of a government commission. 2006.
Ref Type: Report

76. Kerkvliet,N.I. (1995). Immunological effects of chlorinated dibenzo-p-dioxins. Environ Health Perspect 103 Suppl 9:47-53.

77. Kimmel,C.B. (1989). Genetics and early development of zebrafish. Trends Genet 5:283-288.

78. Kleinbaum,D.G. (2000). Survival analysis: a self-learning text. New York: Springer Verlag.

79. Kohno,K. (2007). How Transmembrane Proteins Sense Endoplasmic Reticulum Stress. Antioxid Redox Signal.

80. Kramer,V.J., Helferich,W.G., Bergman,A., Klasson-Wehler,E., and Giesy,J.P. (1997). Hydroxylated polychlorinated biphenyl metabolites are anti-estrogenic in a stably transfected human breast adenocarcinoma (MCF7) cell line. Toxicol Appl Pharmacol 144:363-376.

81. Kuiper,R.V., Bergman,A., Vos,J.G., and van den,B.M. (2004). Some polybrominated diphenyl ether (PBDE) flame retardants with wide environmental distribution inhibit TCDD-induced EROD activity in primary cultured carp (Cyprinus carpio) hepatocytes. Aquat Toxicol 68:129-139.

82. Kuiper,R.V., Canton,R.F., Leonards,P.E., Jenssen,B.M., Dubbeldam,M., Wester,P.W., van den,B.M., Vos,J.G., and Vethaak,A.D. (2007). Long-term exposure of European flounder (Platichthys flesus) to the flame-retardants tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD). Ecotoxicol Environ Saf 67:349-360.

83. Kuriyama,S.N., Talsness,C.E., Grote,K., and Chahoud,I. (2005). Developmental exposure to low dose PBDE 99: effects on male fertility and neurobehavior in rat offspring. Environ Health Perspect 113:149-154.

84. Labandeira,A., Eljarrat,E., and Barcelo,D. (2007). Congener distribution of polybrominated diphenyl ethers in feral carp (Cyprinus carpio) from the Llobregat River, Spain. Environ Pollut 146:188-195.

85. Law,R.J., Allchin,C.R., de,B.J., Covaci,A., Herzke,D., Lepom,P., Morris,S., Tronczynski,J., and de Wit,C.A. (2006). Levels and trends of brominated flame retardants in the European environment. Chemosphere 64:187-208.

86. Layton,A.C., Sanseverino,J., Gregory,B.W., Easter,J.P., Sayler,G.S., and Schultz,T.W. (2002). In vitro estrogen receptor binding of PCBs: measured activity and detection of hydroxylated metabolites in a recombinant yeast assay. Toxicol Appl Pharmacol 180:157-163.

87. Lee,K.-M., Kim,J.-H., and Kang,D. (2005). Design issues in toxicogenomics using DNA microarray experiment. Toxicology and Applied Pharmacology 207:200-208.

88. Li,S., Washburn,K.A., Moore,R., Uno,T., Teng,C., Newbold,R.R., McLachlan,J.A., and Negishi,M. (1997). Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. Cancer Res 57:4356-4359.

89. Lilienthal,H., Hack,A., Roth-Harer,A., Grande,S.W., and Talsness,C.E. (2006). Effects of developmental exposure to 2,2 ,4,4 ,5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats. Environ Health Perspect 114:194-201.

90. Lin,L.L. and Janz,D.M. (2006). Effects of binary mixtures of xenoestrogens on gonadal development and reproduction in zebrafish. Aquat Toxicol 80:382-395.

91. Lin,T.M., Simanainen,U., Moore,R.W., and Peterson,R.E. (2002). Critical windows of vulnerability for effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on prostate and seminal vesicle development in C57BL/6 mice. Toxicol Sci 69:202-209.

92. Longnecker,M.P., Klebanoff,M.A., Zhou,H., and Brock,J.W. (2001). Association between maternal serum concentration of the DDT metabolite DDE and preterm and small-for-gestational-age babies at birth. Lancet 358:110-114.

93. Lundkvist,U. (1990). Clinical and reproductive effects of Clophen A50 (PCB) administered during gestation on pregnant guinea pigs and their offspring. Toxicology 61:249-257.

94. Lyche,J., Larsen,H., Skaare,J.U., Tverdal,A., Dahl,E., Johansen,G., and Ropstad,E. (2004a). Effects of perinatal exposure to low doses of PCB 153 and PCB 126 on lymphocyte proliferation and hematology in goat kids. J Toxicol Environ Health A 67:889-904.

95. Lyche,J.L., Oskam,I.C., Skaare,J.U., Reksen,O., Sweeney,T., Dahl,E., Farstad,W., and Ropstad,E. (2004b). Effects of gestational and lactational exposure to low doses of PCBs 126 and 153 on anterior pituitary and gonadal hormones and on puberty in female goats. Reprod Toxicol 19:87-95.

96. Maack,G. and Segner,H. (2004). Life-stage-dependent sensitivity of zebrafish (Danio rerio) to estrogen exposure. Comp Biochem Physiol C Toxicol Pharmacol 139:47-55.

97. Madsen, L., Lee, S., and Olle, T. Growing threaths: toxic flame retardants and children's health. Environment California Research and Policy Center. 2003.
Ref Type: Report

98. Manchester-Neesvig,J.B., Valters,K., and Sonzogni,W.C. (2001). Comparison of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) in Lake Michigan salmonids. Environ Sci Technol 35:1072-1077.

99. Mandal,P.K. (2005). Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology. J Comp Physiol [B] 175:221-230.

100. Mariussen,E. and Fonnum,F. (2003). The effect of brominated flame retardants on neurotransmitter uptake into rat brain synaptosomes and vesicles. Neurochem Int 43:533-542.

101. McCoy,G., Finlay,M.F., Rhone,A., James,K., and Cobb,G.P. (1995). Chronic polychlorinated biphenyls exposure on three generations of oldfield mice (Peromyscus polionotus): effects on reproduction, growth, and body residues. Arch Environ Contam Toxicol 28:431-435.

102. Meerts,I.A., Letcher,R.J., Hoving,S., Marsh,G., Bergman,A., Lemmen,J.G., van der,B.B., and Brouwer,A. (2001). In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PDBEs, and polybrominated bisphenol A compounds. Environ Health Perspect 109:399-407.

103. Meironyte,D., Noren,K., and Bergman,A. (1999). Analysis of polybrominated diphenyl ethers in Swedish human milk. A time-related trend study, 1972-1997. J Toxicol Environ Health A 58:329-341.

104. Mhanni,A.A. and McGowan,R.A. (2004). Global changes in genomic methylation levels during early development of the zebrafish embryo. Dev Genes Evol 214:412-417.

105. Mills,L.J. and Chichester,C. (2005). Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Sci Total Environ 343:1-34.

106. Morris,S., Allchin,C.R., Zegers,B.N., Haftka,J.J., Boon,J.P., Belpaire,C., Leonards,P.E., Van Leeuwen,S.P., and de,B.J. (2004). Distribution and fate of HBCD and TBBPA brominated flame retardants in North Sea estuaries and aquatic food webs. Environ Sci Technol 38:5497-5504.

107. Morse,D.C., Groen,D., Veerman,M., van Amerongen,C.J., Koeter,H.B., Smits van Prooije,A.E., Visser,T.J., Koeman,J.H., and Brouwer,A. (1993). Interference of polychlorinated biphenyls in hepatic and brain thyroid hormone metabolism in fetal and neonatal rats. Toxicol Appl Pharmacol 122:27-33.

108. Muir,D.C. and Norstrom,R.J. (2000). Geographical differences and time trends of persistent organic pollutants in the Arctic. Toxicol Lett 112-113:93-101.

109. Muirhead,E.K., Skillman,A.D., Hook,S.E., and Schultz,I.R. (2006). Oral exposure of PBDE-47 in fish: toxicokinetics and reproductive effects in Japanese Medaka (Oryzias latipes) and fathead minnows (Pimephales promelas). Environ Sci Technol 40:523-528.

110. Murvoll,K.M., Jenssen,B.M., and Skaare,J.U. (2005). Effects of pentabrominated diphenyl ether (PBDE-99) on vitamin status in domestic duck (Anas platyrhynchos) hatchlings. J Toxicol Environ Health A 68:515-533.

111. Nacci,D., Coiro,L., Champlin,D., Jayaraman,S., McKinney,R., Gleason,T.R., Munns,W.R., Specker,J.L., and Cooper,K.R. (1999). Adaptions of wild populations of the estuarine fish Fundulus heteroclitus to persistent environmental contaminants. Marine Biology 134:9-17.

112. Nakari,T. and Pessala,P. (2005). In vitro estrogenicity of polybrominated flame retardants. Aquat Toxicol 74:272-279.

113. Nilsen,B.M., Berg,K., Eidem,J.K., Kristiansen,S.I., Brion,F., Porcher,J.M., and Goksoyr,A. (2004). Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening. Anal Bioanal Chem 378:621-633.

114. NTP. National Toxicology Program. NTP Technical Report 309, NIH Publication No. 86-2565. 1986. US Department of Health and Human Services. Toxicology and Carcinogenesis Studies of Decabromodiphenyl Oxide (CAS No. 1163-19-5) In F344/N Rats and B6C3F1 Mice (Feed Studies).
Ref Type: Report

115. Orn, S. The zebrafish as a model organism for evaluation of endocrine disrupters. 2006.
Ref Type: Thesis/Dissertation

116. Orn,S., Andersson,P.L., Forlin,L., Tysklind,M., and Norrgren,L. (1998). The impact on reproduction of an orally administered mixture of selected PCBs in zebrafish (Danio rerio). Arch Environ Contam Toxicol 35:52-57.

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

118. Orn,S., Yamani,S., and Norrgren,L. (2006). Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and Japanese medaka after exposure to 17alpha-ethinylestradiol and 17beta-trenbolone. Arch Environ Contam Toxicol 51:237-243.

119. Orn,U. and Klasson-Wehler,E. (1998). Metabolism of 2,2',4,4'-tetrabromodiphenyl ether in rat and mouse. Xenobiotica 28:199-211.

120. PANUPS. EPA revises views on dangerous pesticide lindane. Pesticide Action Network Updates Service. 2006. EPA Tevises Views on Dangerous Pesticide Lindane.
Ref Type: Report

121. Persky,V., Turyk,M., Anderson,H.A., Hanrahan,L.P., Falk,C., Steenport,D.N., Chatterton,R., Jr., and Freels,S. (2001). The effects of PCB exposure and fish consumption on endogenous hormones. Environ Health Perspect 109:1275-1283.

122. Peters,A.K., van,L.K., Bergman,A., Bohonowych,J., Denison,M.S., van den,B.M., and Sanderson,J.T. (2004). Effects of polybrominated diphenyl ethers on basal and TCDD-induced ethoxyresorufin activity and cytochrome P450-1A1 expression in MCF-7, HepG2, and H4IIE cells. Toxicol Sci 82:488-496.

123. Peterson,R.E., Theobald,H.M., and Kimmel,G.L. (1993). Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. Crit Rev Toxicol 23:283-335.

124. Poland,A., Glover,E., and Kende,A.S. (1976). Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. J Biol Chem 251:4936-4946.

125. Poland,A. and Knutson,J.C. (1982). 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu Rev Pharmacol Toxicol 22:517-554.

126. POPRC. Summary of alpha-hexachlorocyclohexane proposal. UNEP/POPs/POPRC.2/15. 2006. Persistent Organic Pollutants Review Committee.
Ref Type: Report

127. Portigal,C.L., Cowell,S.P., Fedoruk,M.N., Butler,C.M., Rennie,P.S., and Nelson,C.C. (2002). Polychlorinated biphenyls interfere with androgen-induced transcriptional activation and hormone binding. Toxicol Appl Pharmacol 179:185-194.

128. QIAGEN. RNeasy Kit; RNA Cleanup. 2007.
Ref Type: Internet Communication

129. R foundation for statistical computing. R development core team. 2004. Vienna, Austria.
Ref Type: Generic

130. Ratcliffe,D.A. (1970). Changes attributable to pesticides in egg breakage frequency and eggshell thickness in some British birds. J Appl Ecol 7:67-115.

131. Reijnders,P.J. (1986). Reproductive failure in common seals feeding on fish from polluted coastal waters. Nature 324:456-457.

132. Remberger,M., Sternbeck,J., Palm,A., Kaj,L., Stromberg,K., and Brorstrom-Lunden,E. (2004). The environmental occurrence of hexabromocyclododecane in Sweden. Chemosphere 54:9-21.

133. Restum,J.C., Bursian,S.J., Giesy,J.P., Render,J.A., Helferich,W.G., Shipp,E.B., Verbrugge,D.A., and Aulerich,R.J. (1998). Multigenerational study of the effects of consumption of PCB-contaminated carp from Saginaw Bay, Lake Huron, on mink. 1. Effects on mink reproduction, kit growth and survival, and selected biological parameters. J Toxicol Environ Health A 54:343-375.

134. Rice,C.P., Chernyak,S.M., Begnoche,L., Quintal,R., and Hickey,J. (2002). Comparisons of PBDE composition and concentration in fish collected from the Detroit River, MI and Des Plaines River, IL. Chemosphere 49:731-737.

135. Ronisz,D., Finne,E.F., Karlsson,H., and Forlin,L. (2004). Effects of the brominated flame retardants hexabromocyclododecane (HBCDD), and tetrabromobisphenol A (TBBPA), on hepatic enzymes and other biomarkers in juvenile rainbow trout and feral eelpout. Aquat Toxicol 69:229-245.

136. Ronnback,C. (1991). Effects of 3,3',4,4'-tetrachlorobiphenyl (TCB) on ovaries of foetal mice. Pharmacol Toxicol 68:340-345.

137. Safe,S. (2001). Molecular biology of the Ah receptor and its role in carcinogenesis. Toxicol Lett 120:1-7.

138. Safe,S.H. (1994). Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. Crit Rev Toxicol 24:87-149.

139. Sager,D.B. and Girard,D.M. (1994). Long-term effects on reproductive parameters in female rats after translactational exposure to PCBs. Environ Res 66:52-76.

140. Sanderson,J.T., Boerma,J., Lansbergen,G.W., and van den,B.M. (2002). Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. Toxicol Appl Pharmacol 182:44-54.

141. Sarkar,A., Ray,D., Shrivastava,A.N., and Sarker,S. (2006). Molecular Biomarkers: their significance and application in marine pollution monitoring. Ecotoxicology 15:333-340.

142. Schecter,A., Cramer,P., Boggess,K., Stanley,J., Papke,O., Olson,J., Silver,A., and Schmitz,M. (2001). Intake of dioxins and related compounds from food in the U.S. population. J Toxicol Environ Health A 63:1-18.

143. Schecter,A., Pavuk,M., Papke,O., Ryan,J.J., Birnbaum,L., and Rosen,R. (2003a). Polybrominated diphenyl ethers (PBDEs) in U.S. mothers' milk. Environ Health Perspect 111:1723-1729.

144. Schecter,A., Quynh,H.T., Pavuk,M., Papke,O., Malisch,R., and Constable,J.D. (2003b). Food as a source of dioxin exposure in the residents of Bien Hoa City, Vietnam. J Occup Environ Med 45:781-788.

145. Schlezinger,J.J. and Stegeman,J.J. (2001). Induction and suppression of cytochrome P450 1A by 3,3',4,4',5-pentachlorobiphenyl and its relationship to oxidative stress in the marine fish scup (Stenotomus chrysops). Aquat Toxicol 52:101-115.

146. Sellstrom,U., Kierkegaard,A., de Wit,C.A., and Jansson,B. (1998). Polybrominated diphenyl ethers and hexabromocyclododecane in sediment and fish from a Swedish river. Environmental Toxicology and Chemistry 17:1065-1072.

147. Siddiqi,M.A., Laessig,R.H., and Reed,K.D. (2003). Polybrominated diphenyl ethers (PBDEs): new pollutants-old diseases. Clin Med Res 1:281-290.

148. Simon,T., Britt,J.K., and James,R.C. (2007). Development of a neurotoxic equivalence scheme of relative potency for assessing the risk of PCB mixtures. Regul Toxicol Pharmacol 48:148-170.

149. Skinner,M.K. (2007). Endocrine disruptors and epigenetic transgenerational disease etiology. Pediatr Res 61:48R-50R.

150. Smith,D. (1999). Worldwide trends in DDT levels in human breast milk. Int J Epidemiol 28:179-188.

151. Spitsbergen,J.M., Schat,K.A., Kleeman,J.M., and Peterson,R.E. (1986). Interactions of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with immune responses of rainbow trout. Vet Immunol Immunopathol 12:263-280.

152. Stapleton,H.M., Letcher,R.J., and Baker,J.E. (2004a). Debromination of polychlorinated diphenyl ether congeners BDE 99 and BDE 183 in the intestinal tract of the common carp (*Cyprinus carpio*). Environ Sci Technol 38:1054-1061.

153. Stapleton,H.M., Letcher,R.J., Li,J., and Baker,J.E. (2004b). Dietary accumulation and metabolism of polybrominated diphenyl ethers by juvenile carp (*Cyprinus carpio)*. Environmental Toxicology and Chemistry 23:1939-1946.

154. Sumpter,J.P. and Jobling,S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect 103 Suppl 7:173-178.

155. The Danio rerio Sequencing Project. http://www.sanger.ac.uk/Projects/D_rerio/. 2007.
Ref Type: Internet Communication

156. The Norwegian Microarray Consortium. http://www.mikromatrise.no. National FUGE-Platform for DNA MicroArray Technology . 2007.
Ref Type: Internet Communication

157. Thomsen,C., Liane,V.H., Frøshaug,M., and Becher,G. (2006). The concentrations of PBDEs in serum from a group of high consumers of fish from a PBDE contaminated lake in Norway. Organohalogen Compounds 68:786-789.

158. Troxel,C.M., Buhler,D.R., Hendricks,J.D., and Bailey,G.S. (1997). CYP1A induction by beta-naphthoflavone, Aroclor 1254, and 2,3,7,8-tetrachlorodibenzo-p-dioxin and its influence on aflatoxin B1 metabolism and DNA adduction in zebrafish. Toxicol Appl Pharmacol 146:69-78.

159. Turusov,V., Rakitsky,V., and Tomatis,L. (2002). Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks. Environ Health Perspect 110:125-128.

160. Uchida,D., Yamashita,M., Kitano,T., and Iguchi,T. (2002). Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. J Exp Biol 205:711-718.

161. Ulbrich,B. and Stahlmann,R. (2004). Developmental toxicity of polychlorinated biphenyls (PCBs): a systematic review of experimental data. Arch Toxicol 78:252-268.

162. van Bakel,H. and Holstege,F.C.P. (2004). In control: systematic assessment of microarray performance. EMBO reports 5:964-969.

163. Van den,B.M., Birnbaum,L., Bosveld,A.T., Brunstrom,B., Cook,P., Feeley,M., Giesy,J.P., Hanberg,A., Hasegawa,R., Kennedy,S.W., Kubiak,T., Larsen,J.C., van Leeuwen,F.X., Liem,A.K., Nolt,C., Peterson,R.E., Poellinger,L., Safe,S., Schrenk,D., Tillitt,D., Tysklind,M., Younes,M., Waern,F., and Zacharewski,T. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106:775-792.

164. van der Ven,L.T.M., Verhoef,A., van de,K.T., Slob,W., Leonards,P.E., Visser,T.J., Hamers,T., Herlin,M., Hakansson,H., Olausson,H., Piersma,A.H., and Vos,J.G. (2006). A 28-day oral dose toxicity study enhanced to detect endocrine effects of hexabromocyclododecane in Wistar rats. Toxicol Sci 94:281-292.

165. Verhaegen,S., McGowan,A.J., Brophy,A.R., Fernandes,R.S., and Cotter,T.G. (1995). Inhibition of apoptosis by antioxidants in the human HL-60 leukemia cell line. Biochem Pharmacol 50:1021-1029.

166. Walker,N.J., Wyde,M.E., Fischer,L.J., Nyska,A., and Bucher,J.R. (2006). Comparison of chronic toxicity and carcinogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in 2-year bioassays in female Sprague-Dawley rats. Mol Nutr Food Res 50:934-944.

167. Watanabe,I. and Sakai,S. (2003). Environmental release and behavior of brominated flame retardants. Environ Int 29:665-682.

168. White,C.A. and Salamonsen,L.A. (2005). A guide to issues in microarray analysis: application to endometrial biology. Reproduction 130:1-13.

169. Whitehead,S.A. and Rice,S. (2006). Endocrine-disrupting chemicals as modulators of sex steroid synthesis. Best Pract Res Clin Endocrinol Metab 20:45-61.

170. WHO. World Health Organisation. DDT and its derivatives, International Programme on Chemical Safety, EHC 9. 1979.
Ref Type: Report

171. WHO. Global assessment of the state-of-the-science of endocrine diruptors. Damstra, T., Barlow, S., Bergman, A., Kavlock, R., and Van Der Kraak, G. 2002. World Health Organization. International Programme on Chemical Safety.
Ref Type: Report

172. WHO. Hexachlorobenzne in Drinking-water. WHO/SDE/WSH/03.04/100. 2004. World Health Organization.
Ref Type: Report

173. Yamazaki,K., Teraoka,H., Dong,W., Stegeman,J.J., and Hiraga,T. (2002). cDNA cloning and expressions of cytochrome P450 1A in zebrafish embryos. J Vet Med Sci 64:829-833.

174. Younglai,E.V., Holloway,A.C., Lim,G.E., and Foster,W.G. (2004). Synergistic effects between FSH and 1,1-dichloro-2,2-bis(P-chlorophenyl)ethylene (P,P'-DDE) on human granulosa cell aromatase activity. Hum Reprod 19:1089-1093.

175. Zhao,G., Xu,Y., Li,W., Han,G., and Ling,B. (2007). PCBs and OCPs in human milk and selected foods from Luqiao and Pingqiao in Zhejiang, China. Sci Total Environ 378:281-292.

176. Zhou,T., Ross,D.G., DeVito,M.J., and Crofton,K.M. (2001). Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. Toxicol Sci 61:76-82.

177. Zodrow,J.M., Stegeman,J.J., and Tanguay,R.L. (2004). Histological analysis of acute toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in zebrafish. Aquat Toxicol 66:25-38.

Appendix 1: Concentrations of POPs

Mean measured concentrations of PCBs, HCHs, HCB, DDTs, chlordanes, BDEs and HBCD in burbot captured from two sourced, Lake Mjøsa and Lake Losna, and in the stock solutions extracted from liver oil from the burbots caught in Lake Mjøsa and Lake Losna. The concentrations are given in ng/g lipid weight (l/w).

	Mjøsa Burbot	Mjøsa Stock	Losna Burbot	Losna Stock
Number (n)	20		21	
Lipid (% mean)	39.3-42.7		48	
Component				
HCB	41	43.8	87	60
HCHs				
α – HCH	5	3.4	4	3.6
β – HCH		12		3
γ – HCH	3.7	2.8	5.1	3.6
Chlordanes				
Oxy-chlordane	30	8.8	11	7.2
Cis-chlordane	28	23	6.8	9.2
Trans-nonachlor	91	75	35	33
DDTs				
pp-DDE	2207	1520	128	141
pp-DDD	161	113	38	46
pp-DDT	912	904	65	66
PCBs				
PCB-28	25	25	7	9
PCB-52	70	40	10	20
PCB-47	25	16	6.4	7.8
PCB-74	48	18	8.9	6.5
PCB-66	73	55	10	9.8
PCB-101	228	166	29	31
PCB-99	178	131	18	17
PCB-110	289	269	27	37
PCB-151	106	75	10	12
PCB-149	72	85	8	16

	Mjøsa Burbot	Mjøsa Stock	Losna Burbot	Losna Stock
Number (n)	20		21	
Lipid (% mean)	39.3-42.7		48	
Component				
PCB-118	341	232	36	34
PCB-153	1270	918	140	150
PCB-105	164	85	11	10
PCB-141	78	52	7.9	7.4
PCB-137	39	27	3.3	3
PCB-138	932	608	89	81
PCB-187	90	58	6.5	9.4
PCB-183	131	83	14	12
PCB-128	129	144	13	40
PCB-156	95	84	10	9
PCB-180	562	334	64	53
PCB-170	198	120	21	18
PCB-189	20	13	2	2.2
PCB-194	60	30	8.5	6
PCB-206	17	19	3.3	2
PBDEs				
BDE-28	206	78	0.8	1
BDE-47	14917	15800	39	46
BDE-100	4572	4300	14	13
BDE-99	8890	8020	40	42
BDE-154	1479	1210	5	4.5
BDE-153	1351	1040	5.5	4.2
HBCD	3379	6060	18	26

Appendix 2: Pilot study

Concentrations and fat percentages in pooled samples [2 fish pooled; ng/g wet weight (w/w) and lipid weight (l/w)] of BDE-47 and PCB-153 in unexposed fish, fish exposed to Losna mixture and fish exposed to two doses of Mjøsa mixture after 10, 20 and 31 days during the pilot study.

	Control		**Losna**					
	31 days		**10 days**		**20 days**		**31 days**	
Fat percent:	10.4		10.3		3.37		2.38	
	w/w	l/w	w/w	l/w	w/w	l/w	w/w	l/w
BDE-47	3.12	30.0	3.32	32.2	3.59	107	3.77	158
PCB-153	16.4	158	14.6	142	13.7	407	15.9	668

	Mjøsa Low					
	10 days		**20 days**		**31 days**	
Fat percent:	7.85		5.94		4.07	
	w/w	l/w	w/w	l/w	w/w	l/w
BDE-47	9.27	118	22.2	374	20.2	496
PCB-153	12.7	162	12.8	215	16.0	393

	Mjøsa High					
	10 days		**20 days**		**31 days**	
Fat percent:	8.63		9.27		4.23	
	w/w	l/w	w/w	l/w	w/w	l/w
BDE-47	51.3	594	62.8	677	124	2927
PCB-153	15.0	174	18.5	200	23.3	552

Appendix 3: Buffers and solutions

Pre-hybridization buffer

Bovine Serum Albumin (BSA) Fraction V	0.1%
SSC	5x
SDS	0.1%

Wash Solution 1

SSC	0.5x
SDS	0.01%

Wash Solution 2

SSC	0.06x

HEPES-Cortland (HC) buffer

$MgSO_4x7H_2O$	0.230 g
KCl	0.375 g
$NaH_2PO_4xH_2O$	0.410 g
NaCl	7.745 g
$CaCl_2x2H_2O$	0.230 g
Hepes	1.429 g
Glucose	1.000 g
Per 1 L dH_2O (pH 7.7)	

Substrate buffer

10 mM Ethoxyresorufin*	70 µl
1 M Dicumarol	35 µl
Per 35 ml HC buffer	

*stock solution: 5 mg ethoxyresorufin dissolved in 2 ml DMSO

Homogenization buffer

Tris-HCl, pH 7.5	50 mM