Evaluating the effects of long term exposure to environmental relevant concentrations of real life mixtures of persistent organic pollutants (POPs) in the zebrafish model.

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Forord

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Contents

Summary

Persistent organic pollutants (POPs) have been widely distributed throughout the world for decades and while the use of some have been phased out, others are still being utilised and their levels are rising in biota. Levels detected in tissue and blood samples have caused for a growing concern for their potential effects on humans and wildlife and many effects studies have been performed. Focus for research has, however, mainly been on high concentrations of single compounds, although it is the realistic levels of real life mixtures of compounds found in the environment that may possess such a threat.

In this study, we have investigated the long term effects of environmentally relevant levels of real life mixtures of POPs using the zebrafish as a biomonitor organism. Burbots (*Lota lota*) from two sites within the same freshwater system in Norway, Lake Mjøsa and Lake Losna, with different history of pollution, were captured and POPs extracted from the liver oil. Zebrafish were exposed indirectly, by exposing their live-food, from start of feeding and until sexual maturation to either the Losna mixture or to one of three dose levels of the Mjøsa mixture. Survival was monitored throughout the experiment while other demographic variables such as growth and sex ratio were evaluated at the time of sexual maturity. Traditional (protein) biomarkers for EROD activity and vitellogenin induction were also measured in addition to organ-specific differences in gene expression pattern using microarray analysis.

Exposure resulted in significant lower survival rate and increased growth of the fish, while a small but significant induction was observed in the EROD activity. Analysis of gene expression patterns revealed small changes in mRNA levels though differences were clearly seen. The microarray assay indicated oestrogenic effects of both of the mixtures, in addition to other endocrine disrupting effects related to the steroid- and thyroid hormones.

We conclude that long term exposure to real life mixtures of POPs caused direct morphological and phenotypic changes and effects systems related to development and reproduction even at low levels found in the environment, and thus may pose a potential health risk for humans and wildlife living in exposed areas or in other ways being frequently exposed to such mixtures of toxins.

1. Abbreviations

γ-HCH	γ-hexachlorocyclohexane
ACE	Angiotensin-converting enzyme
AGT	Angiotensinogen
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
AP1	Activator protein 1
Ar	Argon
Arnt	Ah receptor nuclear translocator
BNF	β-naphthoflevone
CALUX	Chemical activated luciferase gene expression
Cd	Cadmium
CHX	Cyclohexane
Cu	Copper
Cy3	Cyanine 3
Cy5	Cyanine 5
CYP	Cytochrome P450
DAVID	Database for annotation, visualization and integrated discovery
DDD	Dichloro-diphenyl-dichloroethan
DDE	Dichloro-diphenyl-dichloroethylene
DDT	Dichloro-diphenyl-trichloroethan
DES	Diethylstilbestrol
DMSO	Dimethyl sulfoxide
E1	Estrone
E2	Oestradiol

EE2	17 α-ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen receptor
EROD	7-ethoxyresorufin-*O*-deethylase
FDR	False discovery rate
FKBP	FK506 binding protein
GnRH	Gonadotrophin-releasing hormone
GSH	Glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HBCD	Hexabromocyclododecane
HCB	Hexachlorobenzene
Hg	Mercury
HSD	Hydroxysteroid dehydrogenase
Hsp	Heat shock proteins
IVT	*In vitro* transcription
LIB	Laboratory of immunopathogenesis and bioinformatics
limmaGUI	Linear models for microarray data graphical user interface
LOWESS	Locally weighted scatterplot smoother
MIAME	Minimum information about a microarray experiment
MT	Metallothionein
NIAID	National institute of allergy and infectious diseases
NIVA	Norwegian institute for water research
OC	Organochlorine
OH-PCB	Hydroxy polychlorinated biphenyl
PAH	Polyaromatic hydrocarbon

PAPS	3`-phosphoadenosine-5`-phosphosulfate
PBDE	Polybrominated diphenylethers
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
POP	Persistent organic pollutant
RIN	RNA integrity number
SA	Sulphuric acid
Se	Selenium
SE	Standard error
SMO	Sterol-C4-methyl-oxidase
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
SULT	Sulfotransferase
TCDD	Dioxin
TEF	Toxic equivalence factor
TST	Testosterone
Vtg	Vitellogenin
WHO	World health organization
Zn	Sinc
ZP	Zona pellucida

2. Introduction

Persistent organic pollutants (POPs) have been released into the environment for many decades. Some work as powerful pesticides or serve a wide range of industrial purposes, while others are released as unintended by-products of combustion and industrial processes. The Stockholm Convention on Persistent Organic Pollutants define POPs as toxic organic substances that are highly resistant to degradation in the environment, accumulate in the fatty tissue of organisms and have long-range atmospheric transport potential. These characteristics make the POPs hazardous to humans and wildlife all over the world, especially for organisms on top of the food chain, because of their attraction for fatty tissue and thus ability to biomagnify. An enormous amount of POPs have been produced and used over the last 70 years, although many of the 'older' POPs that were popular in the mid 1900's have been phased out of production or even banned and so their levels are declining in biota. However, other POPs are still being produced in vast amounts and their levels continue to rise in the environment. Many studies have investigated the effects of these compounds individually, however, little is known about their toxic potential when combined. Also, not much is known about the possible toxic effects of these POPs when exposure occurs at concentrations that are found in the environment. Thus to get an understanding of the impact of such exposure, it is important to look more deeply into the effects of realistically dosed mixtures.

2.1 An exposure example – Pollution documented in Lake Mjøsa

After the Second World War and up to a few years ago, the freshwater system in and around Mjøsa (Fig. 1) was subjected to a major pollution from agriculture and industry localized in the area, and different POPs, as well as other toxins, were released into the water.

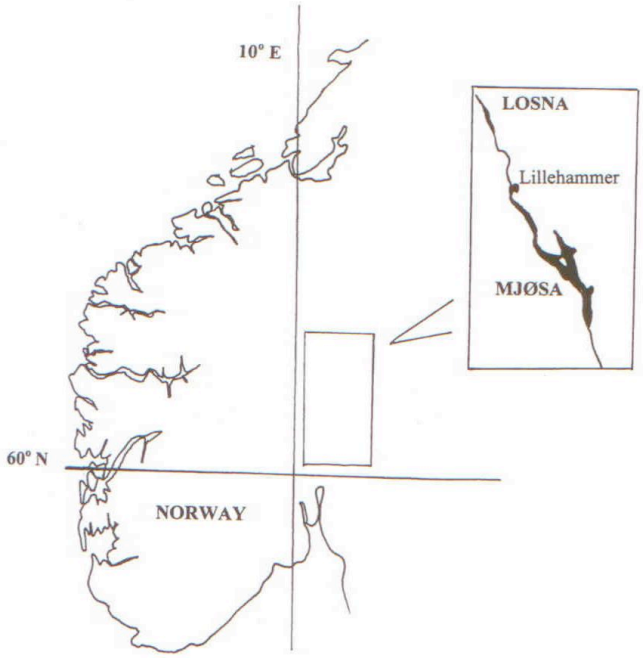


Fig. 1: *Map over southern part of Norway and the location of Lake Mjøsa and Lake Losna. Mjøsa is the largest lake in Norway and Lake Losna is part of the Gudbrandalslågen, which is the major inlet to Lake Mjøsa.*

A study conducted by NIVA in 1999 (NIVA-4072 1999) measured the levels of mercury (Hg), organochlorine (OC) and organotin compounds in fish from Lake Mjøsa with alarmingly results. The levels of mercury in carnivorous fish around 5 years of age ranged between 0,21 – 1,40 mg Hg/kg (w/w), with a mean concentration of 0,64 mg Hg/kg, and 19 of the 24 fish caught contained levels higher than the 0,5 mg Hg/kg limit set for fish to be eaten by the population. Between 1960 and 1970, a cardboard factory in Lillehammer was responsible for spilling mercury into Lake Mjøsa, and although it is more than 30 years since the mercury spills from the wood-processing industry stopped, the levels measured in this study had not decreased significantly since the 80's.

Of the OC compounds measured in fish, the mean concentration of the seven most common congeners of polychlorinated biphenyls (PCBs) (PCB-28, -53, -101, -118, -138, -153 and -180, indicated with PCB7) was 3 762 ng/g lipid weight while it for sum dichloro-diphenyl-tricloroethan (DDT) was 3 053 ng/g lipid weight. These levels are

considerably higher than background levels found in comparable clean environments. Hydraulic- and transformer oil spills from a workshop belonging to the national railway company was responsible for a major PCB pollution during the 60's and 70's, while DDT was extensively used as an insecticide after the Second World War, explaining the high levels of this pesticide. Other OC compounds like γ-hexachlorocyclohexane (HCH), octachlorstyren and penta- and hexachlorbenzen (QCB and HCB) showed only background levels in the fish.

Føsvik and Brevik had already measured the concentration of tributyl tin and triphenyl tin the same year and they found mean levels of 4,8 and 25 ng Sn/g (wet weight) respectively. This was, however, not high enough not to be considered a risk for the environment or people.

In 2003, NIVA conducted a new study (NIVA-4809 2004) where they measured the levels of organic toxins in fish, including PCBs again. This time they found a total PCB7 concentration that ranged between 626 – 1 598 ng/g lipid weight, which was lower than the 1999 recordings and thus indicating a decline in PCB levels. The study also found a mean concentration of polybrominated diphenylethers (PBDEs) of 22 000 ng/g lipid weight in carnivorous fish from Mjøsa, with individual values varying from 7 100 to 45 000 ng/g. Of this, BDE-47, -99 and -100 accounted for between 85 – 90 % of the total PBDEs. The levels of PBDEs in fish have risen continually between 1990 and 2003, and some of the concentrations measured in the study were close to the highest recorded internationally in fish at the time (47 000 ng/g l/w). High levels of Hexabromocyclododecane (HBCD), bisphenol A and chlorinated paraffins were also observed (880, 802 and 2 660 ng/g l/w respectively), indicating the complex pollution scenario that has occurred due to the industry in the area. Levels of PBDEs were also analysed in fish from Lake Losna (Fig. 1), and burbot caught here showed a mean concentration of 49 ng/g lipid weight. This is only about 0,2 % of the levels found in fish from Mjøsa, and Losna is therefore looked upon as a comparable clean environment when considering PBDEs.

The two NIVA studies clearly indicate that fish-eating fish from the Mjøsa area contains a large variety of toxins due to exposure of a great mixture of compounds. This study uses this scenario to investigate the effects of environmental concentrations of real-life mixtures on aquatic organisms.

2.2 Toxins

As indicated above, the exposure that has occurred in the Mjøsa area is very complex and organic compounds and heavy metals are found in addition to several persistent toxins. However, this study seeks to investigate the effects of different POPs in mixture, with the three predominant present being DDT, PCBs and PBDEs, and thus the focus will be aimed here.

2.2.1 DDT, one of the 'old' POPs

A Swiss scientist, Paul Hermann Müller, who later won the Nobel Prize in Physiology and Medicine for his achievement, first recognized DDT as an insecticide in 1939. The discovery led to an enormous increase in the production and usage of DDT, which continued through the 1960´s. It was mainly used in agriculture, to protect the crops and to fight off malaria worldwide, and works by opening sodium ion channels in the insects' neurons and cause them to fire action potentials spontaneously which leads to spasms and eventually death. Some insects, however, have mutations in their sodium channel gene that make them resistant to the chemical.

DDT is created when trichloroethanol reacts with chlorobenzen and three different isomers are made. The p,p'-DDT is the most abundant (app. 85%) isomer and is the only one that work as an insecticide (Fig. 2).

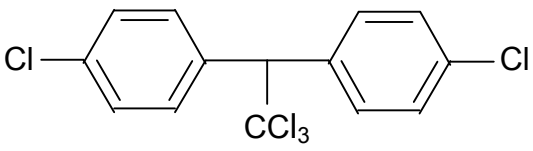


Fig. 2: *p,p'-DDT, the most abundant DDT isomer.*

The o,p'-DDT and o,o'-DDT (have one or both Cl atoms in ortho position) isomers` benzene are easily oxidized, making them less persistent, and they are therefore present at much smaller amounts in the environment.

DDT and its metabolites dichloro-diphenyl-dichloroethylene (DDE) and dichloro-diphenyl-dichloroethane (DDD) have all been shown to induce the phase I biotransformation enzymes cytochrome P450 (CYP) subfamily 2B and 3 (CYP2B and CYP3) and are thus called phenobarbital inducers (Nims et al. 1998). DDE is also shown to be an androgen receptor antagonist, indicating effects on the endocrine system, and some studies have shown an association between DDE blood levels and mammary cancer as reviewed in Turusov et al. 2003. In addition, both DDT and the main metabolites, p,p`-DDE and p,p`-DDD, are shown to be carcinogenic in mice (Turusov et al. 2002).

p,p`-DDT is dehydrochlorinated by the phase II detoxifying enzyme DDT-dehydrochlorinase, which is a glutathione transferase (GST), to p,p`-DDE through a reaction that requires glutathione (GSH). p,p`-DDT can also be converted to p,p`-DDD through reductive dechlorination, and it is evidence that p,p`-DDD can be converted to p,p`-DDE through dehydrochlorination during exposure to high levels of DDT in mammals (Wojtowicz et al. 2004). DDT is also slowly metabolized by micro-organisms in sediments to DDE and DDD. p,p`-DDE is more persistent than p,p`-DDT and it is this metabolite that is found more frequently and at higher levels in humans and animals.

DDT was banned in the US from agricultural use in 1967 following a major debate about its alleged cancer and eggshell thinning effect. Several other uses were banned in 1973, while in Norway DDT was banned in 1970. Some countries in Asia, Africa and Latin America are still using DDT to control diseases like malaria because the public health advantages of using the insecticide are seen as greater than the risks from exposure (Turusov et al. 2002).

2.2.2 PCBs, another of the 'old' POPs

PCBs are organic chemicals that were first synthesized in the 1930´s. There are 209 possible different congeners consisting of a biphenyl with different levels of chlorination, ranging from 1 to 10 chlorine atoms. PCBs are synthesised from biphenyls under high temperature together with Ferric Chloride (FeCl) (Fig. 3), and can be classified according to their number of Cl atoms as described in Table 1.

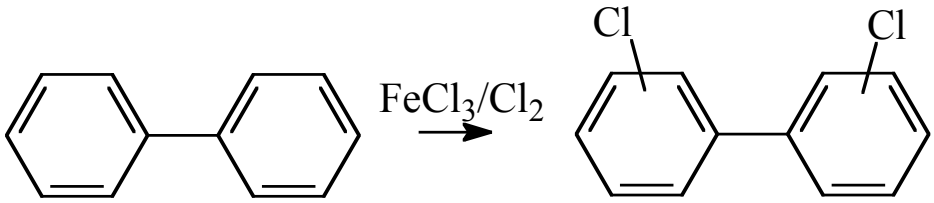


Fig. 3: *Synthesis of PCBs occurs at 350ºC in presence of ferric chloride. The longer the reaction, the more Cl atoms are added to the biphenyl.*

Name	IUPAC nr. (PCB-X)	Molecular formula	Number of congeners	Chlorine content (in %)
Mono	1-3	$C_{12}H_9Cl$	3	18,79
Di	4-15	$C_{12}H_8Cl_2$	12	31,77
Tri	16-39	$C_{12}H_7Cl_3$	24	41,30
Tetra	40-81	$C_{12}H_6Cl_4$	42	48,65
Penta	82-127	$C_{12}H_5Cl_5$	46	54,30
Hexa	128-169	$C_{12}H_4Cl_6$	42	58,93
Hepta	170-193	$C_{12}H_3Cl_7$	24	62,77
Octa	194-205	$C_{12}H_2Cl_8$	12	65,98
Nona	206-208	$C_{12}HCl_9$	3	68,73
Deca	209	$C_{12}Cl_{10}$	1	71,10

Table 1: *Overview over the different PCB congeners, their IUPAC names and distribution between mono and deca congeners. Over half of the congeners are tetra, penta and hexa substituted, while some are too unstable to be detected.*

PCBs are very persistent and highly lipophilic, though they become gradually more soluble in water with higher degree of chlorination (Borja et al. 2005).

PCBs are viscous substances and were thus used as oil in transformers, hydraulic fluids in tools and equipment, dielectrics in capacitors and heat transfer fluids. It was also used as lubricants for turbines and pumps and in some applications as pesticides, inks, dyes and waxes (Borja et al. 2005).

The toxicity of PCBs is linked to their conformation and coplanar congeners are the most hepatotoxic. These are non-ortho substituted and resemble dioxin (TCDD; a polyaromatic hydrocarbon, PAH) in their ability to bind to and induce Aryl hydrocarbon receptor (AhR) mediated responses. The congeners, called PAH inducers, have been shown to affect the immune and reproductive system, induce CYP1A transcription and to be liver toxic. Mono-ortho substituted PCBs show similar effects but are not as potent as the non-ortho. PCBs with minimum two chlorine atoms in ortho positions are not co-planar because of steric strain in the molecule and are called phenobarbital inducers. These have little or no affinity for the AhR and show different toxic effects, such as neurotoxicity, estrogenic activity and induce phase II CYP2B enzymes (Sanchez et al. 2000). A study published in 2005 also showed that the degree of chlorination played a role in the effects on placental steroidogenesis, and lower chlorinated PCBs were shown to decrease oestradiol (E2) secretion, thus having an antioestrogenic effect, while higher chlorinated PCBs increased the E2 secretion and consequently had an oestrogenic effect (Grabic et al. 2006), both showing endocrine disrupting abilities.

Some PCBs, especially the lower chlorinated ones, may be transformed to different metabolites by the organisms defence system and PCBs with only two chlorine atoms are metabolized through hydroxylation by different CYP enzymes and give highly

reactive arene oxide intermediates. Coplanar non-*ortho* congeners, the PAH inducers, are metabolized by the CYP enzymes, CYP1A1 and CYP1A2, while the non-planar di-*ortho* congeners are metabolized by the phenobarbital-induced enzymes CYP2B1 and CYP2B2. Mono-*ortho* substituted congeners are metabolized by both sets of enzymes (Safe 1993). PCBs can also be metabolized in soils and sediments by anaerobe micro-organisms via reductive dechlorination. Mainly, atoms in *para*- and *meta*-positions are removed, which leads to a detoxification since these congeners are the most toxic.

In 1980, all production, import, exports and trade with PCBs and PCB-containing products were banned in Norway. Although the ban came almost three decades ago, PCBs are still present in building materials and in some electronic and electrical equipment today. In the EU, PCBs were banned in 1986 (EU Directive 85/467/EEC).

2.2.3 PBDEs are 'new' POPs

PBDEs are made up of two phenyl rings with different number of bromine atoms attached, connected by an ether bond (Fig. 4).

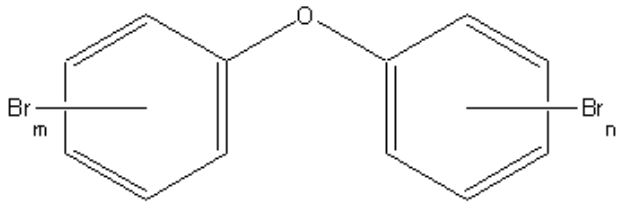


Fig. 4: *The general structure of PBDEs.*

In theory there are 209 possible congeners, although some are too unstable and debrominate to lower brominated congeners. The PBDEs are also divided into groups according to the number of bromine atoms as described in Table 2.

Name	IUPAC nr. (BDE-X)	Molecular formula	Number of congeners	Bromine content (in %)
Tri	17, 28, 33	$C_{12}H_7Br_3O$	3	58,91
Tetra	47, 49, 66, 71, 77	$C_{12}H_6Br_4O$	5	65,79
Penta	85, 99, 100, 119, 126	$C_{12}H_5Br_5O$	5	70,75
Hexa	138, 153, 154, 166	$C_{12}H_4Br_6O$	4	74,49
Hepta	183, 190	$C_{12}H_3Br_7O$	2	77,42
Octa	203	$C_{12}H_2Br_8O$	1	79,77
Nona	206	$C_{12}HBr_9O$	1	81,69
Deca	209	$C_{12}Br_{10}O$	1	83,30

Table 2: *Overview over the different PBDE congeners and their distribution between mono and deca congeners. The penta, octa and deca are the most abundant congeners in the three commercially produced mixtures.*

As with the PCBs, PBDEs are persistent and lipophilic and their water solubility increases with increasing number of bromine atoms.

PBDEs are used as additive flame-retardants, meaning that they are simply blended with polymers and not covalently linked to them as the reactive flame-retardants are. They therefore leak more easily into the environment than some other reactively used brominated flame-retardants like tetrabromobisphenol A.

Of the commercial products produced, pentaBDEs, octaBDEs and decaBDEs are the main components, although they all contain other congeners. The commercial mixture PentaBDE contains the lower brominated BDEs, such as tetraBDEs (30 %), pentaBDEs (55 %) and hexaBDEs (6 %), and BDE-99 and BDE-47 are the main congeners. They account for approximately 75 % of the total mass, and it is about twice as much BDE-99 present as BDE-47, while BDE-153 and BDE-154 are present at approximately equal amounts. In addition they contain some BDE-100. PentaBDE

is mainly used in flexible polyurethane foam, but is also present as flame retardant in phenolic resins, polyesters and epoxy resins. The OctaBDE and DecaBDE mixtures contain higher brominated BDEs, and OctaBDE is used in plastic housing and office equipment, while DecaBDE is used in polystyrene, textiles and electrical and electronic equipment, both as flame retardants (Birnbaum and Hubal 2006). In 2003, PentaBDE accounted for 12 % of the total production of PBDEs, whereas Octa- and DecaBDE products constituted about 6 % and 80 % respectively (Darnerud 2003).

It has been found that PentaBDE in general is more toxic than OctaBDE, while DecaBDE is considered non-toxic to invertebrates (Birnbaum and Hubal 2006). Also, PBDEs have similar structures as PCBs, TCDDs and thyroid hormones (thyroxin T_4 and triiodothyronine T_3) (Fig. 5) and it has been hypothesized that their toxic effects or mode of action might be similar.

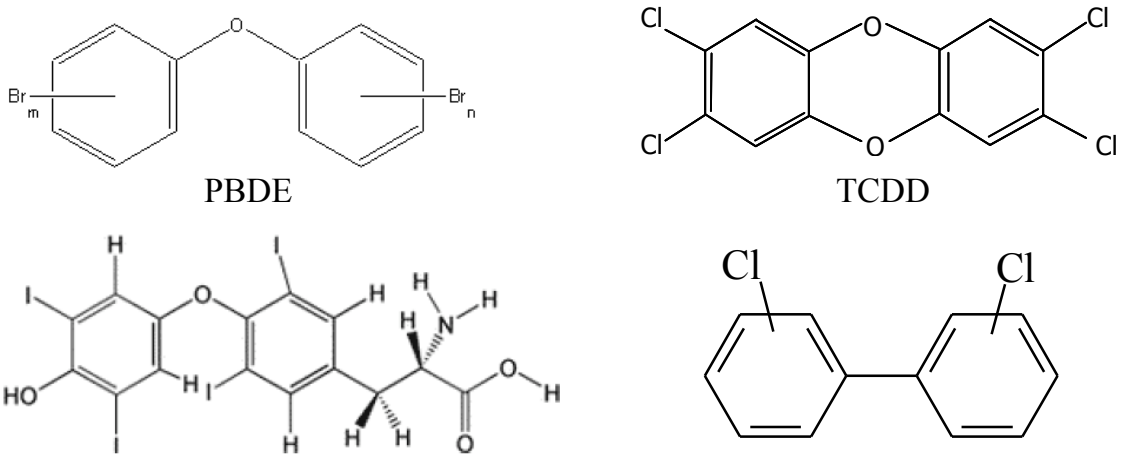


Fig. 5: *Structure similarity of PBDEs and dioxin, thyroxin and PCBs.*

Effects of PentaBDE have been reported on neurobehavioral development and to some extent on thyroid hormones in offspring (Darnerud 2003), and two commercial mixtures, DE-71 (PentaBDE) and DE-79 (OctaBDE) were shown to reduce thyroxine

(T_4) and triiodothyronine (T_3) levels, together with induced phase I CYP1A and CYP2B enzymes, and phase II UDP-glucoronosyl and UDP-glucosyl glucoronosyl enzymes (Zhou et al. 2001). Another study reported that several of the PBDE congeners possessed affinity for the oestrogen receptor and were able to activate oestrogenic responses *in vitro*, though with a much lower potency than E2 (Legler and Brouwer 2003), indicating an effect on the endocrine system. In 2005, (Nakari and Pessala 2005) showed that several of the BDEs have oestrogenic effects by the induced production of vitellogenin in rainbow trout hepatocytes, an induction which increased with higher levels of bromination.

All use of PentaBDE and OctaBDE was banned in Norway and in the EU in 2004 (EU Directive 2003/11/EC), while the use of DecaBDE is restricted in the EU but not in Norway. According to the Stockholm Convention on Persistent Organic Pollutants both PentaBDE and OctaBDE fulfils the requirements of POPs, (are persistent, bioaccumulate, have long-range environmental transport potential and have adverse effects) and are currently under consideration to be included on the list.

The Ah receptor pathway and TEFs

As described above, some PCBs and PBDEs have the ability to induce CYP1A transcription, an enzyme member of the cytochrome P450 oxidase superfamiliy that plays an important role in drug metabolism. This gene subfamily is up-regulated by the induction of the AhR, and is part of the phase I reactions that activate the metabolism of foreign substances. The AhR belongs to the intracellular steroid hormone receptor super family and translocates to the nucleus, when activated by a ligand, where it binds to response elements on DNA and work as a transcription factor (Fig. 6).

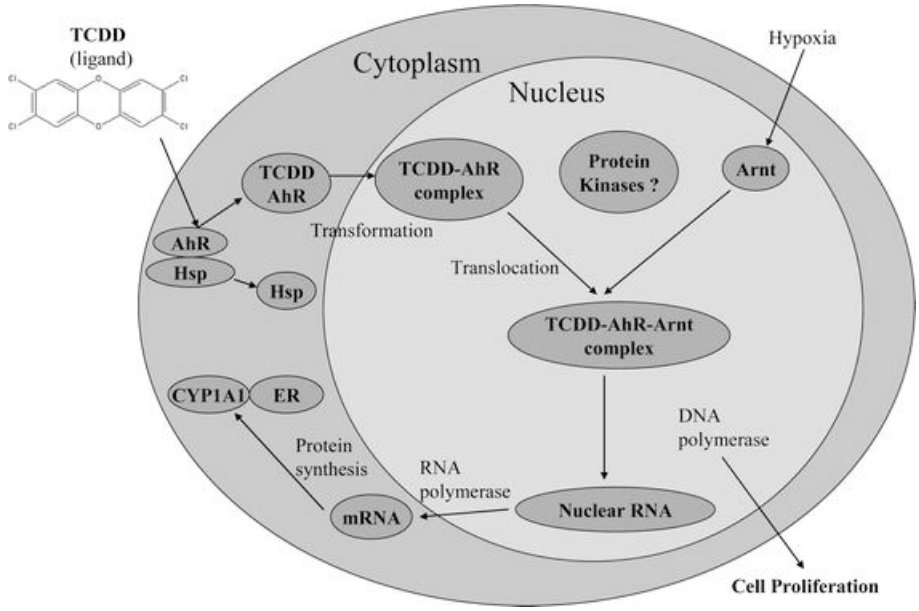


Fig. 6: *The Ah receptor pathway. The receptor is activated by a ligand (in this case dioxin) and the complex travels to the nucleus where it binds to Arnt (Ah receptor nuclear translocator). The complex then binds to XREs (xenobiotic response elements) on DNA and promotes the transcription of response genes like CYP1A. Cytoplasmic AhR is inactivated by Hsp (heat shock proteins). (ER: endoplasmatic reticulum)(Mandal 2005)*

TCDD has the highest affinity for the AhR of the toxic substances and is therefore given a toxic equivalence factor (TEF) of 1. Other compounds are given a TEF of 1 or lower according to their affinity for the receptor and ability to induce AhR-mediated responses compared to dioxin (Ahlborg et al. 1994). According to a list made by the World Health Organization (WHO) in 1997, non-*ortho*, coplanar PCBs (PCB 81, 77, 126 and 169) have TEFs ranging from 0,005 to 0,00005 in fish, while they range from 0,1 to 0,0001 in mammals (Van den Berg et al. 1998). In 2001, (Behnisch et al. 2001) measured the dioxin-like potency of 17 PBDE congeners with the CALUX (Chemical Activated Luciferase gene eXpression) bioassay, where H4IIE cells with a luciferase gene coupled to dioxin response elements as a reporter gene are exposed to chemicals. They found that seven of them were able to activate the Ah receptor in an agonistic (BDE-166 and BDE-190), partly agonistic (BDE-99 and BDE-119) and antagonistic (BDE-85) way. Even though studies like this have shown that some PBDEs can induce CYP1A, they have not yet been put on the WHOs list of TEFs.

2.3 POPs and endocrine disruption

The POPs described have similar chemical properties and are all, as indicated, known as endocrine disrupting chemicals (EDCs) (Pocar et al. 2003),(Legler and Brouwer 2003). The endocrine system mediates information about metabolism, development, growth and reproduction to specific organs of the body, through secretion of hormones from different glands. The most important endocrine glands are the hypothalamus, pituitary and pineal glands in the brain, the thyroid gland in the throat, the thymus in the chest and the adrenal gland, pancreas and ovary/testis in the abdomen. EDCs work by different mechanisms and can mimic the activity of naturally-produced hormones, block receptors that react to the hormones, or hinder the synthesis, secretion, transport and metabolism of hormones (Pocar et al. 2003). A normal hormone balance is crucial for correct development during embryogenesis and is important for proper reproductive function and immune response and in maintaining and regulation of homeostasis in adults (Oskam.I.C 2004). Even though the endocrine system regulates many organ systems, most studies concentrate on chemicals effects on development and reproduction, with focus on the steroid hormones that control this.

Steroidogenesis

Steroid hormones can be classified into five groups according to what receptor they bind to. Glucocorticoids bind to cytosolic glucocorticoid receptors, which are found in the cells of almost all vertebrate tissue. Cortisol is the main glucocorticoid and is involved in metabolism as well as in anti-inflammatory- and immune responses. Mineralocorticoids bind to the cytosolic mineralocorticoid receptor and regulate the salt and water balance. Aldosterone is the most prominent hormone, but the glucocorticoid cortisol also has affinity for this receptor. The enzyme 11 β-hydroxysteroid dehydrogenase type II, present in mineralocorticoid target tissue, catalyze the deactivation of glucocorticoids and thus prevent over-stimulation of the mineralocorticoid receptor. Progestogens bind to the progesterone receptor and continuous secretion of this hormone is essential for preparation and maintenance of

pregnancy, though they also play a role in the luteal phase of the estrus cycle (Oskam.I.C 2004). Progesterone is the natural hormone while synthetically produced progestogens are called progestins. The last two groups of steroid hormones are the sex hormones androgens and oestrogens. Androgens, such as testosterone, bind to androgen receptors and are responsible for development and maintenance of masculine characteristics in vertebrates. Oestrogens, on the other hand, are the primary female sex hormones and are involved in the maturation of oocytes in oviparous vertebrates. E2 and estrone (E) are the two main examples and they bind to the intracellular oestrogen receptor.

All of the steroid hormones are synthesized from cholesterol by a variety of oxidative enzymes shown in Figure 7. The side chain cleavage enzyme (desmolase, CYP11A1) is responsible for converting cholesterol into pregnenolone, which is further oxidized by 3 β-hydroxysteroid dehydrogenase to progesterone. Other important enzymes are CYP11B1 (11 β-hydroxylase) and CYP11B2 (aldosterone synthase) that produce cortisol and aldosterone respectively, and CYP19 (aromatase), which is responsible for producing E2 from testosterone. CYP17 (17 α-hydroxylase/17,20 lyase), CYP21A2 (21-hydroxylase) and 17 β-hydroxysteroid dehydrogenase are also involved in the synthesis pathways.

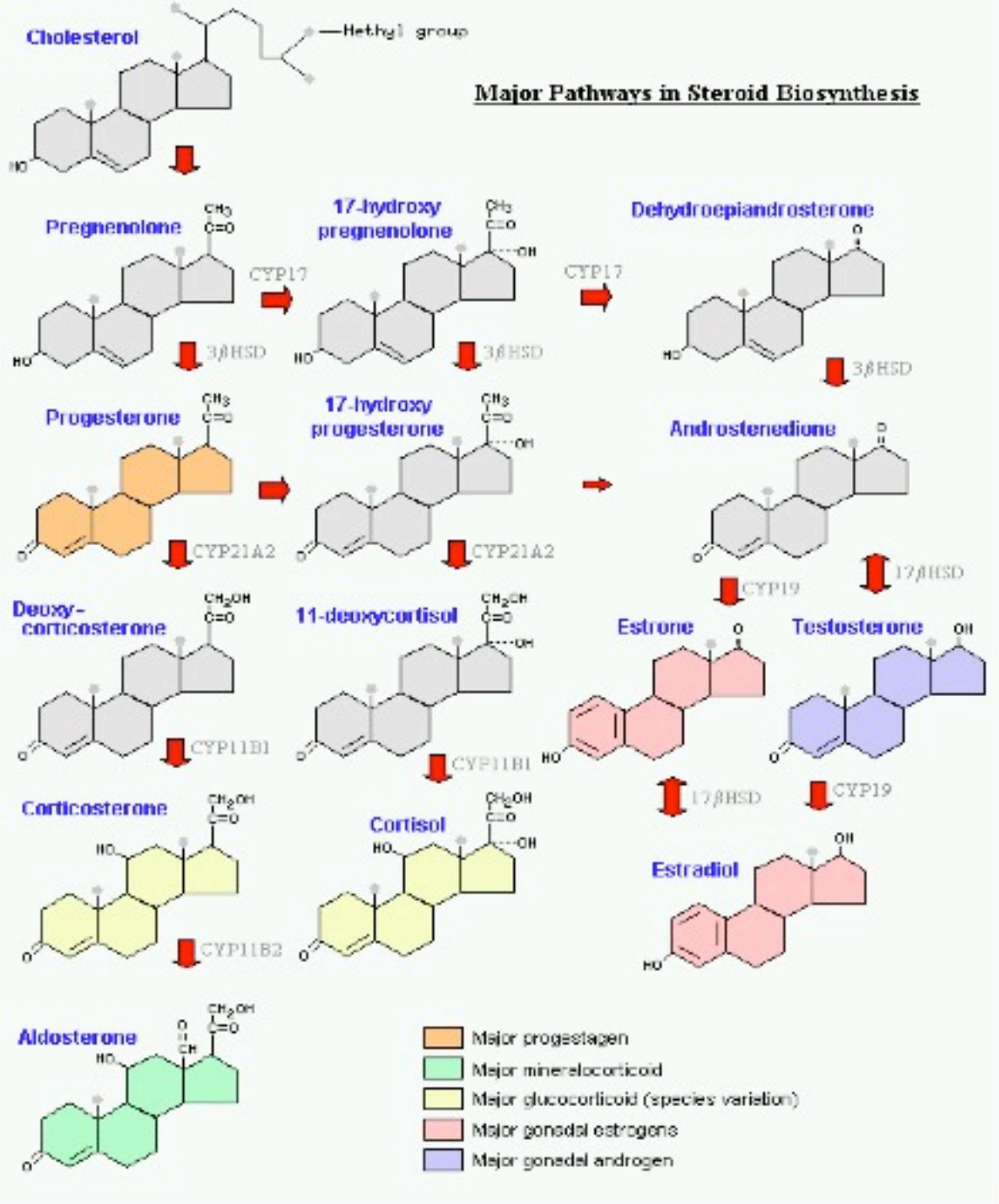


Fig. 7: *Synthesis of the major steroid hormones from cholesterol. The synthesis occurs in mitochondria and enodplasmatic reticulum.*

The endocrine disruptors that affect development and reproduction often target these enzymes involved in steroid hormone production. However, little is known about the possible effects of many such disruptors when they are combined.

2.4 Effects of POPs in mixture

It is clearly that the pollution that has occurred in the Mjøsa area is both complex and extensive, and even though the spillage of many of the older toxins like PCB and DDT has stopped, they are still present inn sediment and fish though the levels are slowly declining. On the other hand, the input of other newer toxins (like PBDEs) continues, explaining the rising levels observed. As indicated above, much is known about the individual chemicals and their toxic potential, but their combined effects on the environment and population around are not known. Addition, antagonism and synergism are well known concepts in toxicology, and describe some possible interactions between compounds. An additive interaction is an independent joint action where compounds toxicity is not affected by the other chemicals present, and the effects can be 'added' together. A similar joint action, on the other hand, refers to compounds that have similar mode of action and can heighten (agonise) or lessen (antagonise) each other's toxic effect. These two forms of interactions can to some extent be predicted by knowing the toxicity of the individual components present. A synergistic action however, can not be predicted in the same way by the individual ingredients since knowledge about the chemicals combined toxicity is needed, making it virtually impossible to accurately foresee the toxic effects of combined complex mixtures (Monosson 2005) and at what levels they will have an effect. In spite of this, over 95 % of all toxicological studies performed up to 1995 focused only on single chemicals (Feron et al. 1995) and in recent years, single groups of chemicals. Of the few studies that do look at the interaction between chemicals, it is usually the effects of only two compounds combined that are investigated (Eriksson et al. 2006) and (Kang et al. 1996). However, we are seldom exposed to single chemicals as they are preset all around us every day, and it is therefore a huge combination of compounds, like observed in the Mjøsa area, that possess an exposure risk to humans and wildlife and that must be investigated. Also, to further evaluate the realistic exposure risk, it is important to investigate possible effects using environmental relevant concentrations. The lack of studies published that approach this problem indicate that there is clearly a

need for evaluating the toxic effects of real life mixtures and the Mjøsa scenario is an excellent example.

2.5 Zebrafish as a vertebrate model

The zebrafish (*Danio rerio*) is a small fresh water fish naturally occurring in the tropic areas of south Asia. It has a life span of approximately two years, is 3-4 cm long and has a generation time of three to four months. They are sexually mature for ~1,5 years and the females lay between 100 and 300 eggs weekly, which are externally fertilised by the males. The embryos are transparent, develop quickly and hatch after two days. They inflate their swim blather at around five days post fertilisation (5dpf) thus becoming free-swimming larvae that are able to take in food (Alestrom et al. 2006) and (Westerfield 2000).

The above characteristics make the zebrafish well suited as an experimental animal. Their small size makes them easy and cheap to keep in the laboratory and the short generation time is a huge advantage in multiple generation studies. The zebrafish have mainly been used to study vertebrate developmental due to the high egg production, transparent embryos and a well characterized early development. Eggs and larvae can easily be monitored and physical defects observed under a microscope. In 2001, the Sanger Institute (Cambridgeshire, England) launched the zebrafish whole-genome sequencing project and 5th of April 2006 the zebrafish assembly version 6 (Zv6) was released (Sprague et al. 2006). It comprises a total sequence length of 1.626.077.335 bp in 6.653 fragments where 1.547.299.723 bp of these are placed on chromosome 1-25. The zebrafish genome is predicted to contain more genes than the human genome because of a genome duplication that occurred in ancestors approximately 400 million years ago. Some of the duplicated genes have evolved to get new functions while others were lost, but many are still present and every fifth mammalian gene has in fact two zebrafish orthologs (Dahm and Geisler 2006). With the genome being sequenced and more genes annotated, the zebrafish is gradually becoming a popular model in other areas of biology too, such as in toxicology and functional genomics. For

instance, assays specific for zebrafish have been developed for measuring common biomarkers indicating exposure to different toxin groups while morpholino-mediated knockdown of genes is becoming a well-established method in zebrafish that can be used to study the function of selected genes. With a sequenced genome several microarray systems are commercially available and make it possible to perform different gene expression analysis, which is very informative in functional genomics and toxicogenomics studies (reviewed in (Hill et al. 2005), (Alestrom et al. 2006) and (Dahm and Geisler 2006)).

2.6 Investigating the results of exposure

2.6.1 Biomarkers

Biomarkers are widely used in toxicology to indicate exposure to certain toxins, and several assays are validated, well described and easy to perform. Two of the most common molecular markers are EROD activity (Whyte et al. 2000) and vitellogenin (Sumpter and Jobling 1995).

EROD

The 7-ethoxyresorufin-*O*-deethylase (EROD) assay indicates whether the toxins present have dioxin-like attributes by indirectly measuring CYP1A induction *in vitro* or *in vivo*. In fish, EROD activity is traditionally measured in hepatic microsomes (Mdegela et al. 2006) however this method requires several steps of tissue preparation and may not be the most sensitive since pollutions are readily metabolised in the liver. CYP1A transcription is, however, also induced in the gills and a fast and sensitive method for measuring EROD activity in gill filaments was developed by (Jonsson et al. 2002).

Burke and Mayer (1974) first described the method of using EROD activity as a marker for hepatic microsomal activity. 7-ethoxyresorufin work as a substrate and CYP1A catalyze the O-dealkylation reaction that produces the product resorufin.

Resorufin is a pink fluorescent dye that emits light upon excitation and is detected by a fluorometer. With an excess number of substrate molecules, the amount of resorufin produced is proportional to the amount of CYP1A enzyme present. If an organism or cell has been exposed to TCDD or dioxin-like compounds that activate the AhR and induce CYP1A transcription, it is detected by an increase in resorufin levels.

Vitellogenin

Vitellogenin (Vtg) is an egg yolk precursor protein that is only expressed in the liver of sexually mature female oviparous vertebrates in response to oestrogen. Males and immature females have very low plasma levels of the protein. The hypothalamus secretes gonadotrophin-releasing hormone (GnRH), which stimulates the pituitary to produce and secrete gonadotrophin hormones I and II. These hormones stimulate the immature ovary to release E2 into the bloodstream and the increase in circulating E2 indirectly causes the ovary to produce Vtg. In liver cells, E2 activate the oestrogen receptor (ER), which binds to oestrogen response elements on DNA located in the promoter region of E2 inducible genes and causes an increase in transcription, including of Vtg mRNA. Vtg is then transported in the bloodstream to the ovary where it is incorporated in oocytes and processed.

In toxicology, the detection of Vtg in males is used as a biomarker for exposure to different xenoestrogens and when E2 is administered to males, or immature females, a dose-responsive increase in Vtg plasma levels occurs. Even low doses of E2 can result in a million-fold increase of Vtg, making this a very sensitive marker for oestrogen exposure. To stimulate Vtg expression, xenoestrogens must be able to bind to the alpha subunit of the ER and their affinity for the receptor varies. Some compounds, like alkylphenols and some halogenated organic compounds and pesticides, have low affinity for the ER and are thus only weakly oestrogenic. Other produced chemicals, like 17 α-ethinylestradiol (EE2) and diethylstilbestrol (DES), are more potent than E2 itself. Also, there is an additive interaction between oestrogens so an increase in Vtg levels can occur even though the compounds independently would not have this effect.

In 2001, (Holbech et al. 2001) published a method using enzyme-linked immunosorbent assay (ELISA) to measure Vtg on zebrafish whole body homogenates and today kits are commercially available to perform this assay in several different species.

2.6.2 Gene expression analysis

In addition to studying the effects of mixtures of POPs on single marker genes, a genome wide approach to learn more about what molecular mechanisms of toxicity and metabolic pathways that are affected was conducted. This was made possible with the use of microarray technology, which measures and compares the transcriptomes from a treated (f.ex. exposed with ecotoxins) biological sample and an untreated sample. Obtaining good, significant results from such assays, however, requires careful planning of the experiment. The Microarray Gene Expression Data Society have therefore come up with the "Minimum Information about a Microarray Experiment" (MIAME) guidelines for reporting and publishing of microarray experiments which help to standardize the assays (Brazma et al. 2001).

There are two main types of microarrays, cDNA (Expressed sequence tags, ESTs) and oligonucleotide (oligo), and they are both commercially available. cDNA libraries exist for members of many phyla and arrays can be made without knowing the gene sequences. Oligo arrays, on the other hand, are made from the coding regions of genes and are only available for species with sequenced genomes. In this study, long oligo arrays designed for the zebrafish model were used.

Long oligo arrays consist of between 50 and 70 bases, and are designed to define a representative of each target gene as predicted using a bioinformatic analysis of the entire genome. In the present expression libraries, each oligo represents one gene, of which the genes can be of known or unknown function, and is usually defined within the 3' exon of the gene, which is often shared between alternatively spliced transcripts. The oligos are also computed to have standardized hybridization conditions and are

thus similar in GC content. Long oligos show more sequence specific binding, which lower the chance for random cross-hybridization (Barrett and Kawasaki 2003).

Long oligo arrays also allow for direct comparisons between samples by labelling them with different fluorescents and hybridizing them on the same slide. Competitive binding to the oligos results in different colour intensities and the differences in mRNA levels in the two samples can be identified (Fig. 8) (White and Salamonsen 2005).

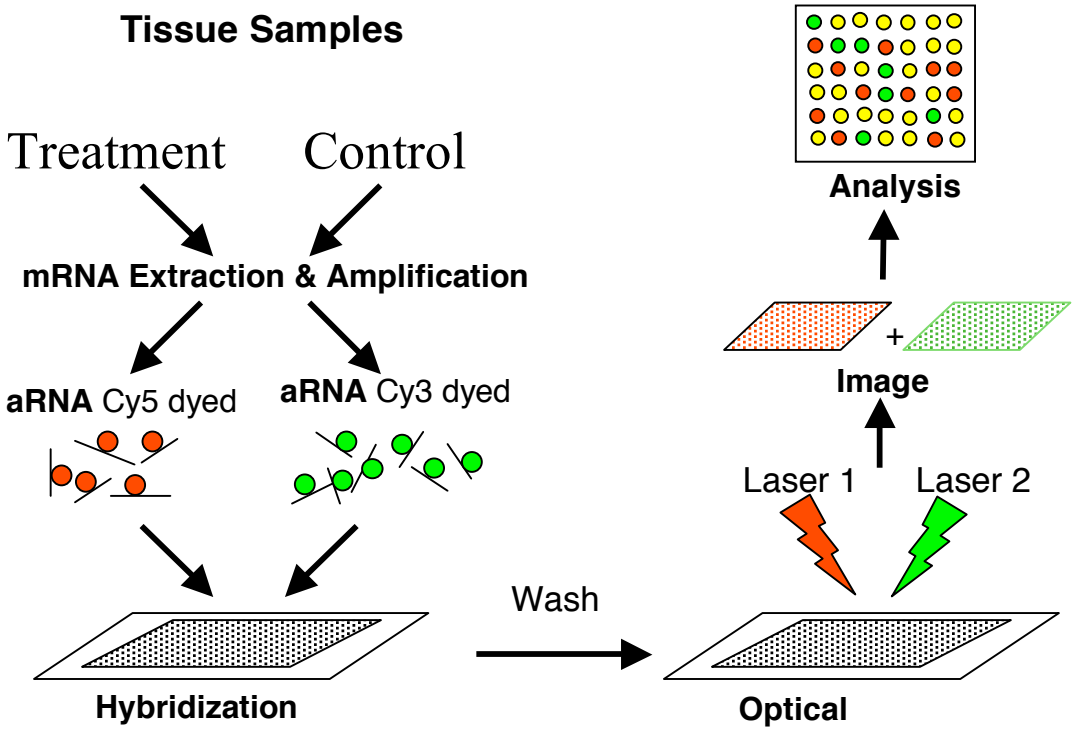


Fig. 8: *Microarray experiment: Amplified, isolated mRNA (aRNA) from treated and reference samples are coupled to different dyes and hybridized onto the slide. Competitive binding gives differences in colour intensity in each spot, representing up or down regulated genes. A scanner is used to detect the dyes and software computes the raw intensity data.*

There are many sources of variation in a microarray experiment that must be considered in order to get a significant result, and they can be divided into technical and biological sources. Technical variation may arise from differences in the tissue

sampling, extraction and processing of mRNA, hybridization and in the printing of the arrays, and can be overcome by standardizing procedures and using replicates. Both oligos and arrays can be replicated and a dye swap replicate is usually included to account for non-biological differences in colour intensities (White and Salamonsen 2005). Biological variation occurs within species and will always be present in different degrees, however, a good assay design can minimize its effect. The use of sufficient number of replicates, or pooling several individuals, reduces biological variation and helps to achieve statistically significant results.

Image acquisition refers to the scanning of the arrays, and it is the fluorescent labels incorporated into the samples that are detected. Cyanine 3 (Cy3, green) and Cyanine 5 (Cy5, red) are widely used as fluorescent dyes today. The conversion of images into numerical values is referred to as image quantification and results in intensity values ranging between 0 and 65536 pixels computed by the software that is used with the scanner. This raw data are imported into programs that compute M and A values from them, and a scatter plot can be made. M and A values are logarithmic transformation of the ratio of the colour intensities ($M=\log_2(R/G)$,R = red and G = green) and the total intensities of the two colours in each spot ($A=1/2\log_2(R*G)$). Possible non-biological effects as described earlier can be seen in the M-A plot as non-linear log-ratios (M-values), and are in most cases related to the technical differences in the print-tip that produced each spot. These differences can be taken into account by adjusting the data means or variances through normalisation, and is done with a LOWESS (LOcally WEighted Scatterplot Smoother) function which adjusts every value individually based on its intensity level and work as a local linear regression analysis that 'straightens' out the data (Forster et al. 2003).

If the experiment involves only one categorical factor, for example treatment vs. non-treatment, the statistical analysis can be performed using an unpaired *t*-test. This tests the null hypothesis that the two samples have equal mean expression levels. It is, however, important to remember the aspect of multiple testing when assigning statistical significance to a large number of genes since P-values are designed for single tests. For instance, a P-value of 0,05 means that 1 out of 20 tests (20*0,05 = 1)

is a false positive, so if 10 000 genes are tested simultaneously, (10 000*0,05 = 500) 500 of these would be false positives which is 20 % of the genes tested. To deal with this problem P-values have to be adjusted to become stricter. For example, an original P-value of 0,05 when testing 10 000 genes can be adjusted so that the new P-value of (0,05/10 000 = 5*E-6) 0,000005 would give the same significant 1 false positive out of 20 tests as above ((10 000*20)*0,000005 = 1). Another method is to calculate the False Discovery Rate (FDR) developed by Benjamini and Hochberg (Benjamini and Hochberg 1995) and adjust the P-values accordingly. The FDR gives the number that is expected to be false positives out of the results, so if, with an original P-value of 0,01, 500 out of 25 000 genes show expression differences, (25 000*0.01 = 250) 250 of these 500 genes are expected to be false positives, a FDR of 50 %. Thus, the P-value can be adjusted so that the number of genes expected to be false positives is much smaller than the number of genes that were actually differently expressed. Adjusted P-values that give a FDR between 1 % and 10 % is normally considered acceptable for publications (Breitling 2006).

After obtaining differently expressed genes with microarray technology, it is considered necessary to verify at least some of the results using quantitative real-time PCR (qRT-PCR) technology or other independent methods. qRT-PCR is a modified PCR amplifying system where probes are hybridized to the products for detection and quantification. Primers are constructed for selected gene transcripts and are used to amplify these during the PCR reaction. Probes can be unspecific or made specific and emits light for detection. Post-translational control, however, is an important step in regulating the proteome and it is therefore common to also verify the results at the protein level with for example western blot analysis.

The microarray system used in this thesis is a 16k 65-mer library printed on slides and made available to the scientific community by the AlestromLab and the Norwegian Microarray Consortium (www.microarray.no).

2.7 Aims of study

The main objective for this thesis was to investigate the effects of long term exposure to environmentally relevant concentrations of real life mixtures of POPs isolated from a polluted Norwegian fresh water system on zebrafish exposed in the laboratory via POP contaminated food.

Fish were exposed to mixtures of POPs for six months, from larva stage and until they reached sexual maturity, at which time the sampling took place for analysis and a second generation was established.

Demographic endpoints such as survival rate of the parent and second generation, sex ratio and growth was evaluated in addition to induction of specific biomarkers and changes in gene expression patterns. We expected to learn more about the indirect zebrafish exposure and the effectiveness of exposing the live feed, toxin metabolism in zebrafish and possible critical windows of exposure. Through the organ-specific genome wide RNA profiling experiments we also expected to learn more about the overall differences in gene expression patterns, the two mixtures impact on the different organs and which cellular processes and pathways are affected by the treatment.

3. Materials and Methods

3.1 Obtaining real life mixtures of POPs

3.1.1 Extraction of mixtures of ecotoxins and chemical assessment

Livers from Burbots (*Lota lota*) weighing 1-2 kg, caught in Lake Mjøsa and Lake Losna (Fig. 1, 61º 02' N, 10º 27' E and 61º 24' N, 10º 13' E respectively) in August 2004, 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 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 (Table 3). BDEs and HBCD were determined by GC-MS according to a method described by (Murvoll et al. 2005). PCBs, HCHs, HCB, chlordanes and DDTs were determined by 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 to 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 artemia and zebrafish after exposure.

3.1.2 Preparation of the Mjøsa and Losna treatments

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 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 cyclohexan from the acid and generating a 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 leas two days for the SA to separate from the organic phase. The CHX was replaced with acetone 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 tree different dose levels of the Mjøsa treatment. Aliquots of the Losna and Mjøsa High treatments were analyzed for POPs (Table 3) with detection limits similar to the burbot analysis.

3.1.3 Measured toxins in burbot livers and the extraced mixtures

The 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 Table 3. 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 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 a little lower.

	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

Table 3: *Mean measured concentrations of PCBs, HCHs, HCB, DDTs, chlordanes, BDEs and HBCD in burbot (Lota lota) 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).*

3.2 Zebrafish (*Danio* rerio)

Wild type zebrafish (*Danio rerio*) previously bought from a pet store were used to give rise to the exposed generation.

3.2.1 Living conditions

Water for the fish was made up by running normal tap water through a reverse osmosis machine and then adding synthetic sea salt (Instant Ocean, Marineland), Sodium Bicarbonate (Sigma), Calcium Sulphate (Sigma) and HCl (Sigma) to get the desired physicochemical properties. The pH was kept at 7,5, the conductivity at around 500 µS/cm and the general and carbonate hardness at about 3ºdH. In addition, the ammonia, nitrate and nitrite levels were monitored every week and kept between the healthy ranges. The water was heated to 28ºC, while the humidity in the room was between 60 % and 70 % and there was a light cycle of 13 hour light/11 hour night. Fish where kept in a Marine Biotech fish flow-through system with bio filters for growth of bacteria culture and carbon filter and UV radiation of the water. A 10 % water change in the system was conducted daily. (More detailed descriptions can be obtained at www.alestromzebrafishlab.no).

3.2.2 Feeding regime

Larvae were fed four times a day, at 9am, 11am, 1pm and 3pm, seven days a week. Either Camilla Almås or I conducted all feedings, with the help of other members of the project some weekends and holidays.

Baby powder as start fodder

Fish were given baby powder food of type SDS 100 (Scanbur A/S, Nittedal, Norway) the first three weeks, from 6-25dpf. The feed was administered with a spatel and ¼ of a spatel was given to each tank at every feeding.

Artemia nauplii (Brine shrimp)) as live food

Artemia are species of aquatic crustaceans that are commonly used as fish food because of their high contents of lipids and unsaturated fatty acids. They are bought as dormant, metabolically inactive eggs and hatch when soaked in salty water. To hatch the artemia (Scanbur A/S, Nittedal, Norway), we followed the manufacturer's protocol which recommended a salt concentration of 28 ppt (parts per trillion, ~35 g/L), an

artemia concentration of max 10 g/L, temperature of 28°C, air-bobbles for movement and a hatching time of 24 hours. We started out making up a volume of 200 ml and gradually increased it as the fish grew and needed more food. Six flasks (one for each group) of new artemia was made up every morning and after 24 hours the water was filtered off, fresh water added and shell-remains removed. The artemia was administered with disposable plastic pipettes and all the tanks were given the same amount, measured in millilitres on the pipettes. As the fish grew larger, the amount was increased. Larvae were given artemia twice a day from on the second week onwards (13-18dpf). During week three (19-25dpf) artemia was given three times a day, and after 25dpf artemia was given as the only feed.

3.3 Pilot study

A pilot study was conducted to get a better understanding of the bioaccumulation of the mixtures in artemia and the biomagnification from artemia to the fish. In addition, the exposure route of the xenoestrogen 17α-ethinylestradiol (EE2, through water or feed) was also evaluated. Adult wild type zebrafish were divided in five groups, Mjøsa High, Mjøsa Low, Losna, EE2 exposed through food and EE2 exposed through water, with ten male and female fish in each group. The fish were fed artemia treated with different levels of toxins.

BDE-47 was used as an indicator and all mixture concentrations were made up based on this congener. The high Mjøsa group was exposed to a concentration of 4 ng/ml artemia, while the low Mjøsa and Losna groups were exposed to 0,5 ng/ml and 0,026 ng/ml respectively. The two last groups were treated with EE2, one through the fodder and the other through water. The food exposed fish received a concentration of ethinylestradiol of 2,5 µg/ml artemia while the concentration for the water exposed group was 1 ng/L. The fish were exposed for one month, and two fish from each group were sampled on day 10, 20 and 31 for analysis.

3.4 Experimental design

To get the best possible understanding of mixture exposure and the differences between Mjøsa and Losna mixture exposure it was decided to expose the F_0 generation for three different doses of the Mjøsa mixture, low, medium and high, while only one high dose of the Losna mixture was prepared. In addition to a solvent control, we also wanted a positive control that was given EE2 because we expected to see oestrogenic effects of the mixtures. For the F_1 generation it was decided to have additional groups, and only the solvent control, the Losna and the Mjøsa High groups were continued with because of lack of space. From the F_0 control fish, F_1 Control-Control, Control-Losna and Control-Mjøsa groups were established, while from the F_0 Losna and Mjøsa High fish F_1 Losna-Control and Losna-Losna and Mjøsa-Control and Mjøsa-Mjøsa group were made. The second part of the names indicated what treatment the F_1 generation received.

15 pairs of fish (one female and two males) were set up for breeding in the afternoon and allowed to mate the following morning, at onset of light. The eggs were collected, counted and taken care of for two days. Dead eggs were removed each day. On the second day, all eggs/hatchlings were grouped together, mixed and randomly divided into batches of 70 eggs/hatchlings each. Five batches corresponded to an exposure group consisting of five replicates (A-E). The groups, Control, Losna, Mjøsa Low, Mjøsa Medium, Mjøsa High and EE2, were named according to the mixture and dose they were to receive. On 6dpf, all batches were adjusted to contain 65 larvae. Later on, the F_1 generation was generated from only the Control, Losna and Mjøsa High groups and each group was divided in several subgroups after which mixture their parents were exposed to and which they themselves were to get (Fig. 9). On 6dpf the numbers of larvae were adjusted to 85. Larvae were kept in small ¾ L tanks that were gradually exchanged with 1 L, 2 L, 4 L and finally 6 L tanks as the fish grew. At 14dpf the tanks were connected to the water flow in the system.

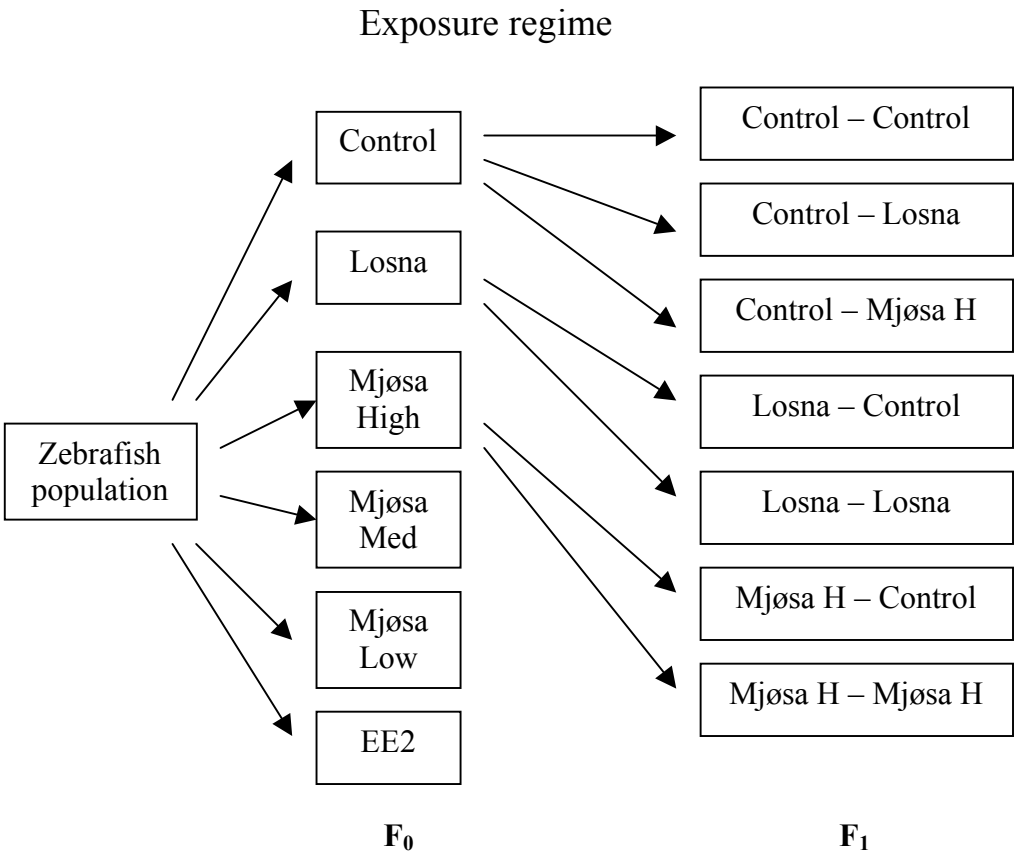


Fig. 9: *Overview over the different exposure groups. All groups, both in the F_0 and F_1 generation, consisted of 5 replicates. Only the Control, Losna and Mjøsa High groups gave rise to the F_1 generation.*

The survival of the fish was monitored throughout the experiment, and to determine whether the fish had become sexual mature, we placed small open boxes with marbles in each tank, which were inspected every day for eggs. When all tanks (all five replicates in all groups) had given eggs, we assumed that most of the fish were sexually mature and the sampling could take place. The fish reached sexual maturity after five months and sampling took place shortly after for four different assay types, toxin determination, histopathology, biomarker detection and gene expression analysis and growth and sex ratio evaluated. One female and one male from each replicate were sampled for toxin determination, while four females and males were sampled for histopathology examination (results not part of this study). Two males from each

replicate were sampled and used in both EROD and vitellogenin analysis, while six of each sex were sampled and used in the microarray assays. In total, eleven female fish and thirteen male fish were sampled from each replicate in all groups. The remaining fish in each tank were used in breeding experiments and to establish the second generation (Fig. 10).

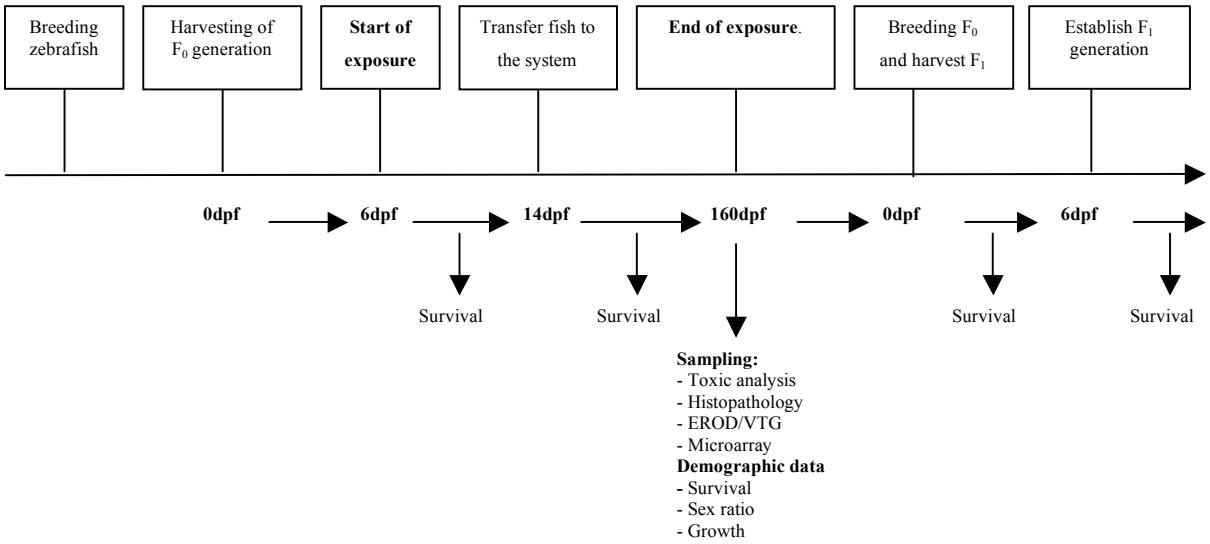


Fig. 10: *Flow diagram of the experiment. The survival was monitored continuously while the growth and sex ratio was evaluated at the same time as sampling took place at around 160dpf.*

3.5 Exposure

The POPs treated fish were exposed through the fodder during the whole experiment. Treated food was administered Monday through Friday, while unexposed food was given in the weekends. Because of the length of the experiment, we assumed that the fish would accumulate wanted levels of the POPs without having to expose them seven days a week. We chose to expose the EE2 group through water because this is a well described method and eliminated the risk of contamination of the other groups because these fish were kept in a separate room.

3.5.1 Exposure through feed

We wanted the exposed fish to accumulate levels of POPs that were in the same order of magnitude as the levels observed in burbot from Lake Mjøsa and Lake Losna.

Treatment of the baby powder

Five batches of baby food labelled Losna, Mjøsa Low, Mjøsa Medium and Mjøsa High were prepared with POPs to expose the zebrafish during the first period. The baby food was analysed and found to contain very low levels of the toxins (below 1 ng/g wet weight of all components), and had a lipid content of 10 %. The food was prepared by adding enough extracts from the Mjøsa and Losna mixture to obtain the same (lipid weight) contamination as observed in the burbot liver oil. These were labelled Mjøsa High and Losna, while 1/8 and 1/32 amount of Mjøsa extract was used to prepare the Mjøsa Medium and Mjøsa Low food respectively. A sixth batch was labelled Control and received only solvent. Abundant CHX was used, covering the food to ensure an even distribution within the baby powder. All batches were then defumed until under N_2 until steady weight was observed.

Treatment of the Artemia nauplii

From the experience obtained during the pilot study, we expected that such level would be achieved if the fish were given artemia hatched in a 250 ml flask with 4000 ng of BDE-47. The mixture extracts were diluted in acetone and to avoid concentrations of acetone exceeding 0,1 %, 250 µl of stock solution was used to administer toxins to the artemia flasks. The Mjøsa extracts was adjusted, through evaporation, to contain 4000 ng/0,250 ml = 16 000 ng/ml BDE-47, making up the Mjøsa High treatment, and control analysis showed that the level was close the what calculated (15 800 ng/g, Table 3). The Losna extract was prepared in the same way as the Mjøsa High treatment, while Mjøsa Medium and Mjøsa Low was prepared by diluting Mjøsa High 1:8 and 1:32 respectively with acetone. Five batches of artemia labelled Control, Losna, Mjøsa Low, Mjøsa Medium and Mjøsa High were made up 250 µl of the different treatments/solvent was added to the corresponding cultures

each morning, exposing the artemia for 24 hours during hatching. A sixth flask labelled Untreated was also made up with no treatment or solvent added, and given to the EE2 water-exposed fish.

3.5.2 Exposure through water

The EE2 group was not kept together with the other groups in the flow-through system but was instead kept in a separate room in water baths heated to 28°C. EE2 solved in acetone was added to a reservoir tank that was filled to 11 L with water from the flow-through system where the other groups were placed, and the fish were given new treated water from this reservoir tank every day. The nominal concentration of EE2 was 10 ng/L and a stock solution of EE2 was made up for convenience.

Procedure:

1. 8 mg of EE2 was weighed and mixed in 1 ml of acetone to give a concentration of 8 mg/ml.
2. 0,1 ml of this was mixed with 99,9 ml of acetone to give 8 µg/ml.
3. 0,1 ml of this was mixed with 9,9 ml of acetone to give a concentration of 0,08 µg/ml which made up the stock solution.

Exposed water was then made up by adding 125 µl of the stock solution per litre of water to the reservoir tank, an up to 90 % of the water in the tanks was replaced with freshly made EE2 water from the reservoir tank to maintain the water quality.

3.6 Recording of demiographic variables

3.6.1 Survival of the F_0 and F_1 generation

The survival of the first generation was monitored until sampling took place (163dpf) by counting the number of dead larvae or fish removed each day and recording it in a

logbook. The survival of the second generation is still being recorded as the fish have not yet reached sexual maturity.

3.6.2 Sex ratio of the F_0 generation

At 161dpf, the sex of the fish was determined by visual inspection. Female fish have a small, white triangle between their ventral fin and tail fin, which is a retrogonadal cavity, and identification of this was used in the assessment.

3.6.3 Growth of the F_0 generation

Fish that were sampled for the different assays were all measured and weighted and the length and weight was recorded. The data was then compared between the groups.

3.7 Toxin determination

One male and one female fish from each replicate were caught and euthanized with an overdose of tricaine methanesulfonate (MS 222, Sigma) in ice water for toxin analysis. Fish were measured and weighted before whole body samples were processed as described in 3.3.1. Artemia was also sampled and analysed.

3.8 Biomarker assays

From all groups, two male fish were collected from each replicate (A-E) and euthanized with an overdose of tricaine methanesulfonate (MS 222, Sigma) in ice water for biomarker assays. From the oestrogen group, female fish were collected. Each fish were weighed and measured before the gills were dissected out for use in EROD analysis. The rest of the fish was wrapped in labelled freeze-foil and freezed immediately in liquid nitrogen and stored at -80ºC for later vitellogenin assay.

3.8.1 EROD analysis

For each fish, three gill arches were dissected from the gills, placed in different wells on a 24 well plate and washed with freshly made HEPES-Cortland buffer. After 1 min, the buffer was removed and replaced with 0,5 ml of EROD buffer (HEPES-Cortland buffer containing 10 mM 7-etoxyresorufin (Sigma) and 1 mM Dicoumarol (Sigma), an anticoagulant). After 2 min, the substrate solution was removed and replaced with new 0,5 ml of EROD buffer and the time point was noted. After ~ 60 min the time point was noted and the substrate solution was transferred to eppendorf tubes and stored at -20°C.

The substrate solutions were later analysed on the Victor plate reader (Perkin Elmer, Massachusetts, USA) located at the National Veterinary Institute. A standard curve with a concentration of 0-16,315 ng resorufin (Sigma) /ml was made using dH_2O. 200 µl of each standard and sample, samples in triplets, was transferred to wells on a 96 wells plate and read on the Victor machine using a protocol for reading EROD that was made in the Workshop software with excitation at wavelength 530 nm and emission at wavelength 580 nm. Three plates, all containing standards, were used to measure the samples and the software automatically calculated the means of the triplets. Data was then transferred to excel and a new standard curve was made using the mean of the three recorded. For each fish, the mean of the three gill arches was manually calculated and compared to the new standard curve to get the concentration of resorufin produced. The obtained data was expressed in pmol resorufin/gill arch/min.

Morten Sandvik, at the department of toxicology at the National Veterinary Institute, made the 7-ethoxyresorufin stock and the buffers.

To make 10 mM 7-ethoxyresorufin, 5 mg was solved in 2 ml of DMSO to give a concentration of 2,5 mg/ml. With a molecular weight of 241,24 g/mol, this equals 10,36 mM of ethoxyresorufin.

3.8.2 Vitellogenin analysis

The freezed whole body samples, lacking gills, were weighed and homogenized in homogenization buffer at a 1:10 relationship. The samples were then centrifuged at 12 500 rpm at 4ºC for 1 hour, before the supernatant below the fat layer was collected in cryo tubes and stored at -80ºC.

The "Quantitative Zebrafish Vitellogenin ELISA test kit" (Biosense) was used to measure the Vtg precursor protein in the fish. The test uses specific binding between antibodies and vitellogenin to quantify the protein in samples. First, one plate was run with one sample from each group in four dilutions (1:11, 1:550, 1:550 000 and 1:1 650 000) to test which dilutions gave results within the standard curve. Then all the samples were run again in a second run with.

Procedure (all reactions supplied by the kit and all steps performed according to the protocol):

1. The content of 1 vial of Vtg standard (4,5 µg) was dissolved in 1 ml of cold Dilution buffer giving a Vtg concentration of 4,5 µg/ml in the stock solution.

2. The first dilution step for the standard curve was prepared by diluting 50 µl of the stock solution in 1 750 µl of cold Dilution buffer to give a concentration of 125 ng Vtg/ml. From this, a two-fold serial dilution was made including 11 dilution steps ranging from 0,12-125 ng/ml

3. The fish homogenates were already diluted 11 times and 5 µl of this was added to 245 µl of cold Dilution buffer to give the 1:550 dilutions. 5 µl of this was added to 4 995 µl to give the 1:550 000 dilutions and 100 µl of this was added to 200 µl to give the 1:1 650 000 dilutions. All dilutions were kept on ice until use.

Incubation with standards and diluted samples

1. 100 µl was added to each of the two NSB (non-specific binding) wells.

2. 100 µl of each standard was added in duplicates.
3. 100 µl of each sample was added in duplicates.
4. Plate was sealed and incubated at RT for 1 hour.

Incubation with Detecting antibody

1. 31 µl of the Detecting antibody was added to 11 ml of Dilution buffer to give a 1:350 dilution.
2. Each well in the plate were washed 3 times with 300 µl of Washing buffer.
3. 100 µl of diluted Detecting antibody was added to all wells before sealing the plate and incubation at RT for 1 hour.

Incubation with Secondary antibody

1. 6 µl of the Secondary antibody was added to 12 ml of Dilution buffer to give a 1:2 000 dilution.
2. Each well in the plate were washed 3 times with 300 µl of Washing buffer.
3. 100 µl of diluted Secondary antibody was added to all wells before sealing the plate and incubation at RT for 1 hour.

Colour development

1. Each well in the plate were washed 5 times with 300 µl of Washing buffer.
2. 100 µl of Substrate solution was added to each well before incubation in the dark at RT for 30 min.
3. 50 µl of 2M H_2SO_4 was added to each well to stop the reaction and the absorbance was read after 5 min.

The absorbance was read at 490 nm with the Victor plate reader (Perkin Elmer, Massachusetts, USA) using a protocol for reading vitellogenin, which was made in the

Workshop software. The software automatically calculated the means of the duplicate standards and samples. Data was then transferred to excel and a new standard curve was made taking the means of the $\log_{10}$ values of the measured absorbances and concentrations of the two standards. The sample concentrations were then calculated using the curve formula and the measured absorbances, and values were given in ng/ml.

3.9 Microarray analysis

Six female fish were collected from each replicates (A-E) of all the groups, and euthanized with an overdose of tricaine methanesulfonate (MS 222, Sigma) in ice water for microarray analysis. Each fish were measured and weighted before the ovary and liver were dissected out. Organs from one fish per replicate in each group were pooled together, however, only ¼ of each ovary was used to get the right amount of tissue for RNA isolation. Brains were also dissected out but not enough total RNA could be isolated from them to do microarray analysis. In total, we obtained six pooled liver samples and six pooled ovary samples from each group containing organs from five different fish each. All tissue samples were freezed immediately after dissection in liquid nitrogen and stored at -80ºC. The sampling was performed over a period of seven days when the fish were 163 to 169 days old. Only the Mjøsa High, Losna and Control groups were further analysed in this study (Fig. 11).

Design:

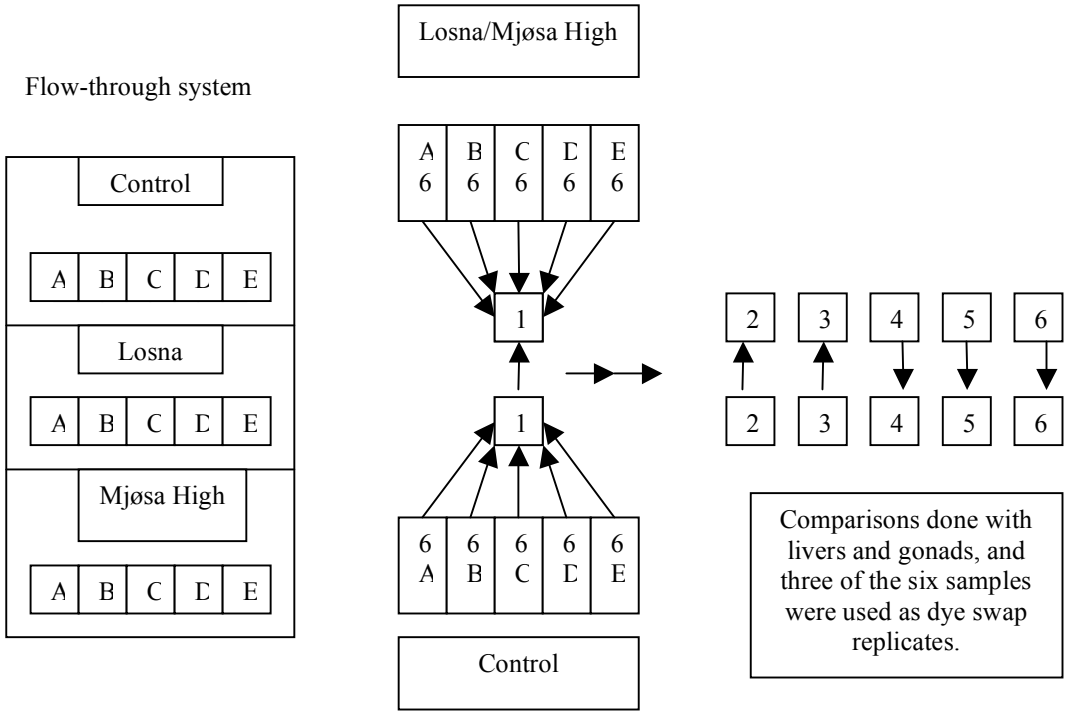


Fig. 11: *Design of the microarray experiments. Ovary/liver from one fish per replicate was pooled (pool 1-6). In three of the pooled samples, mRNA from controls treated fish were labelled with Cy3 and Cy5 respectively, while in the other three pooled samples, mRNA from controls and treated fish were labelled with Cy5 and Cy3 respectively, making dye switch replicates.*

For each organ all pooled samples from the two treated groups Losna and Mjøsa High were compared with the corresponding pooled samples from the control group.

3.9.1 Total RNA preparation

RNA isolation from frozen tissue was performed using Trizol (Invitrogen) and "RNeasy kit" (Quiagen) following the manufactures protocol. The "NanoDrop® ND-1000 spectrophotometer" (NanoDrop Technologies, Delaware, USA) was used to determine the concentration of the isolated RNA. The NanoDrop® covers the full spectrum (220-750 nm) and measures the absorbance of DNA, RNA, proteins, dyes and microbial cell culture OD. Only 1 µl of sample is needed and the accompanied

software automatically calculates the yield, and the 260/280 nm and 260/230 nm ratios. When measuring nucleic acids, these ratios indicate the purity of the sample. A pure sample has a 260/280 nm ratio of ~ 1,8 when measuring DNA while it is closer to 2,0 for RNA. The 260/230 nm ratio should be between 1,8 and 2,2, and lower levels may indicate contaminants present in the sample.

Procedure, isolation with Trizol:

1. 1 ml of Trizol was added to the frozen tissue so that the ratio tissue:trizol was 1:10.

2. Samples were transferred to tubes containing magnalizerbeads (Roche) and homogenized for 50 sec, on frequency 25 (Retsch MM301, Haan, Germany). The containers were turned and homogenized again.

3. 200 µl of chloroform (Merck) was added to the homogenized samples before centrifugation at 4 000 rpm for 15 min in 2-8°C.

4. 400 µl of the water phase was transferred to new eppendorf tubes containing 400 µl of isopropanol (Arcus), incubated for 10 min at RT and centrifuged at 4 000 rpm for 10 min in 2-8°C.

5. The supernatant was removed and the pellet washed with 1 ml of 75 % ethanol (Arcus). Samples were vortexed to loosen pellet and centrifuged at 4 000 rpm for 5 min in 2-8°C.

6. The supernatant was removed and the pellet dried for 5 min.

7. 50µl of RNase free water (Ambion) was added to the pellet before incubation in water bath at 65°C for 2-3 min. Samples were vortexed to dissolve the pellet.

The RNA concentration was checked on the NanoDrop® (in ng/µl).

Procedure, RNeasy kit (all reagents, except β-merkaptoethanol (KEBO Lab) and ethanol (Arcus), were supplied by the manufacturer and all steps were performed according to the protocol):

1. Maximum volume of sample was calculated from the NanoDrop results (the kit columns takes max. 100 µg of RNA) and added to new eppendorf tubes. RNase free water was added to make the volume 100 µl.
2. 350 µl of prepared RLT buffer (consisting of 10µl β-merkaptoethanol/ml of RLT buffer) was added to each sample and mixed.
3. 250 µl of 96 % ethanol was added and samples shaken.
4. The lysates were transferred to filtration columns and spun at 8 000 rpm for 15 sec.
5. Flow-through was discarded and samples spun again at 8 000 rpm for 15 sec.
6. Columns were transferred to new collection tubes, 700 µl of RPE buffer added and samples centrifuged at 8 000 rpm for 15 sec.
7. Flow-through was discarded and samples spun again at 10 000 rpm for 1 min.
8. The columns were transferred to new eppendorf tubes and 50 µl of RNAse free water was added to the membranes. Samples were left for 2-3 min.
9. Columns were centrifuged at 10 000 rpm for 1 min to eluate the purified RNA. Columns were removed and samples put on ice.

The RNA concentrations were checked on the NanoDrop® again and each sample was aliquoted into three tubes, two with equal volume for array analysis and one with 1-2 µl for degradation check. All samples were stored at -80°C.

To ensure good integrity of the RNA in the samples, the “2100 Bioanalyzer” (Agilent Technologies, California, USA) machine was used together with “RNA 6000 Series II Nano kits” (Agilent). 12 samples can be analysed on one 16-wells chip, which

contains separation channels that are filled with a gel containing an intercalating fluorescent dye. Samples are loaded into the wells and one by one injected into the corresponding separating channel where the components are electrophoretically separated. The accompanied software, "2100 expert", calculates the ratio between 28S and 18S rRNA and shows results in a gel-like image as well as in electropherogram and tabular formats. It also calculates a RNA integrity number (RIN-number), which is based on the entire electrophoretic profile of the RNA sample. The result is a number between 1 and 10 where 10 represent intact RNA. We accepted a RIN number of 8 or better when selecting samples for the microarray assay. Some samples did not produce a RIN number and we then compared the samples' electrophoretic profiles with others that had a high RIN number.

Procedure (all reagents were supplied by the manufacturer, and all steps were performed according to the protocol):

Preparing the Gel

1. 550 µl of RNA 6000 Nano gel matrix was transferred to a spin filter and spun at 1 500 g (3 000 rpm) for 10 min (used within 4 weeks).

Preparing the Gel-Dye Mix

2. 65 µl of the filtered gel was transferred to an RNase-free eppendorf tube.

3. The RNA 6000 Nano dye concentrate was equilibrated to room temperature, vortexed for 10 s and spun down.

4. 1 µl of the dye was added to the filtered gel, vortexed well and spun at 13 000 g (~10 000 rpm) for 10 min at RT.

Loading the Chip

5. A new RNA chip was put on the Chip Priming Station.

6. 9 µl of gel-dye mix was loaded onto the chip according to the manufacturer manual.

7. 5 µl of RNA 6000 Nano Marker was added to all 12 sample wells and the ladder well.

8. 1 µl of ladder was added to the specified ladder well.

9. 1 µl of sample was added to each of the 12 sample wells.

10. The chip was vortexed for 1 min and run on the Bioanalyzer within 5 min.

3.9.2 mRNA amplification, dye coupling and hybridization

RNA amplification was performed using the "Amino Allyl MessageAmp™ II aRNA Amplification Kit" with Cy™3 and Cy™5 dyes (Ambion). It produces cDNA from mRNA with the use of an oligo(dT) primer, a reverse transcriptase and a T7 DNA polymerase. The cDNA strands are then used as templates for the *in vitro* transcription (IVT) and the result is multiple copies of antisense amplified RNA (aRNA). During transcription, a modified nucleotide 5-(3-aminoallyl)-UTPs are incorporated into the aRNA. This nucleotide contains a reactive primary amino group on the C5 position of uracil that easily couples to the N-hydroxysuccinimidyl ester-derivatixed reactive dyes (NHS ester dyes) Cy3 and Cy5 supplied with the kit.

Oligonucleotides obtained from the Zebrafish Oligo Library (Cat#XEBLIB384, Sigma Genosys) were printed on glass slides by The Norwegian Microarray Consortium. The slides contained 16 399 oligos, each consisting of 65 bases, and represent 16 288 LEADS clusters (genes) and 171 controls. The LEADS software platform clusters and assembles known mRNAs transcripts.

Procedure RNA amplification (all reagents used in this procedure, except 96 % ethanol (Arcus), were supplied by the manufacturer and all steps were performed according to the protocol):

Reverse Transcription to Synthesize First Strand cDNA

1. Calculated amounts of diluted RNA samples, and RNase free water if needed, were transferred to sterile eppendorf tubes.

2. 1 µl of T7 Oligo(dt) primer and 1 µl Spike-T solution (R solution for control samples) was added to each sample.

3. After incubation for 10 min at 70°C in a thermalcycler, the tubes were briefly spun down and placed on ice.

4. During incubation, a Reverse Transcription Master Mix was prepared in RT, resuspended and briefly centrifuged (volumes are for one sample):

Component	Amount in µl
10x first strand buffer	2
Ribonuclease inhibitor	1
dNTP mix	4
ArrayScript	1
Total	8

5. 8 µl the master mix was added to each sample, mixed and incubated at 42°C for 2 hours in a thermocycler.

6. After incubation, the samples were briefly spun down and placed on ice.

Second Strand Synthesis

1. At the end of incubation, a Second Strand Master Mix was prepared on ice, resuspended and briefly spun down (volumes are for one sample):

Component	Amount in µl
Nuclease-free water	63
10x second strand buffer	10
dNTP mix	4
DNA polymerase	2
RNase H	1
Total	80

2. 80 µl of the master mix was added to each sample, mixed and incubated at 16°C for 2 hours in a thermocycler.

Double Stranded cDNA Clean up

1. During the incubation, an eppendorf tube with nuclease-free water was preheated to 55°C.

2. 250 µl of cDNA Binding Buffer was added to each sample and mixed.

3. The total volume was transferred to cDNA Filter Cartridges (supplied by the manufacturer) and centrifuged for 1 min at 10 000 g. Flow through discarded.

4. 500 µl of Wash Buffer (with ethanol added) was applied to each cDNA Filter Cartridge and spun for 1 min at 10 000 g. Flow through discarded and samples spun again for 1 min at 10 000 g.

5. cDNA Filter Cartridges were transferred to cDNA Elution Tubes (supplied by the manufacturer), and 18 µl of preheated nuclease-free water was added to the centre of the filter in the Cartridges.

6. After 2 min of incubation at RT, samples were centrifuged for 1 min at 10 000 g and cDNA Filter Cartridges removed. If needed, nuclease-free water was added to the eluated samples to make the total volume 14 µl.

Construction of aRNA

1. An IVT Master Mix was prepared in RT, resuspended and briefly centrifuged (volumes are for one sample):

Component	Amount in 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

2. 26 µl of the master mix was added to each sample, mixed and incubated at 37°C for 16 hours in a hybridization oven.

3. After incubation, 60 µl of nuclease-free water was added to the samples to stop the reaction.

aRNA purification

1. Eppendorf tube with nuclease-free water was preheated to 55°C.
2. 350 µl of aRNA binding Buffer was added to each sample.
3. 250 µl of 96% ethanol was added to each sample and mixed.
4. The total volume (700 µl) was transferred to aRNA Filter Cartridges (supplied by the manufacturer) and centrifuged for 1 min at 10 000 g. Flow through discarded.
5. 650 µl of Wash Buffer was added to each aRNA Filter Cartridge and spun for 1 min at 10 000 g. Flow through discarded and samples spun again for 1 min at 10 000 g.
6. aRNA Filter Cartridges were transferred to new aRNA Collection tubes and 100 µl of preheated nuclease-free water was added to the centre of the filter.
7. After 2 min incubation in RT, samples were spun for 1,5 min at 10 000 g and aRNA Filter Cartridges removed.

Sample concentrations were checked on the "NanoDrop ND-1000 spectrophotometer" and stored at -80°C.

Procedure dye coupling (all reagents used in this procedure were supplied by the manufacturer and all steps were performed according to the protocol):

Amino Allyl Coupling

By coupling both dyes to the treated samples and controls so that three of the six samples got one colour and three got the other colour we performed a dye switch control.

1. Calculated volumes of aRNA were transferred to new tubes and Speedvaced until completely dry.

2. 11 µl of DMSO was added to the Cy3 and Cy5 dye tubes supplied, mixed and vortexed, and kept in the dark until use (within one hour).

3. 9 µl of coupling buffer was added to the dried samples and mixed.

4. 11 µl of the prepared DMSO/Cy dye mixes was added to the samples so that the samples which were to be compared got different colours, and incubated for 60 min at RT in the dark.

5. 4,5 µl of Hydroxylamine was added to each sample and vortexed before incubation for 15 min at RT in the dark.

6. 5,5 µl of nuclease-free water was added to each sample to make the total volume 30 µl.

Labelled aRNA Purification

1. RNase free water was preheated to 55°

2. 105 µl of aRNA Binding Buffer was added to all the samples.

3. 75 µl of ACS grade 95 % ethanol was added and mixed by pipetting 3 times.

4. The total volume was transferred to Labelled aRNA filter cartridges (supplied by the manufacturer), and centrifuged for 1 min at 10 000 g. Flow through discarded.

5. 500 µl of aRNA Wash Buffer was added to filters and spun for 1 min at 10 000 g. Flow through discarded.

6. Samples were centrifuged again for 1 min at 10 000 g to remove trace amounts of ethanol.

7. Cartridges were transferred to new Labelled aRNA Elution Tubes (supplied by the manufacturer), and 50 µl of preheated water was added to the centre of the filters.

8. After 2 min incubation at RT, samples were centrifuged for 1,5 min at 10 000 g and aRNA Filter Cartridges removed. Samples were put on ice.

The "NanoDrop ND-1000 spectrophotometer" was then used to measure the aRNA concentration (in ng/µl) and the amount of dye incorporated (in pmol/µl). The mRNA was measured at 260 nm, while Cy3 was measured at 550 nm and Cy5 at 650 nm. To calculate total amount of dye incorporated, pmol/µl of the two dyes were multiplied with the eluted volume.

Procedure hybridization:

Hybridization

1. Slides were incubated in 0,2 µm-filtered pre-hybridization buffer at 42°C for 30-60 min. Before use, they were washed twice with nuclease-free water and once with isopropanol. After last wash, slides were quickly transferred to empty falcon tubes and spun dry in a centrifuge at 1 000 rpm for ~3 min.

2. The total hybridization volume was 450 µl and the maximum volume of the samples was 225 µl. The corresponding Cy3 and Cy5 labelled samples were mixed together and nuclease-free water added to make the volume 216 µl.

3. 9 µl of 25x fragmentation buffer (Agilent) was added to samples before incubation for 30 min at 60°C.

4. Per sample, 4,5 µl of sonicated herring sperm DNA (10µg/µl, a blocking agent) (Promega) was added to 220,5 µl of 2x hybridization buffer (Agilent).

5. 225 µl of 2x hybridization buffer (with DNA) was added to each sample, making a total volume of 450 µl.

6. Samples were spun for 1 min at 13 000 rpm and applied to gasket slides (Agilent) that were placed in hybridization chambers. The printed slides were put on top of the gasket slides and held in place with chamber covers that were fastened with a tightening devise. The flow of the hybridization solution was inspected by slowly rotating the champers to ensure that no bubbles were stationary before placing the chambers in a hybridization oven at 60°C for 16-20 hours with a rotation of 3 rpm.

Washing

1. Three 50 ml falcon tubes were filled with 0,2 µm-filtered Wash solution 1 and two 50 ml falcon tubes were filled with 0,2 µm-filtered Wash solution 2 for each sample.

2. After hybridization, the printed slides, with gasket slides, were transferred to falcon tubes with wash solution 1 where the gasket slides were removed. The tubes were rotated to keep the slides from drying and wrapped in tin foil to keep the samples from bleaching.

3. After 5 min, the slides were transferred to new tubes with wash solution 1 and incubated with agitation in the dark.

4. After additional 5 min, slides were transferred to the last tubes with wash solution 1 and incubated with agitation in the dark. After 5 min, the slides were washed in the same way with wash solution 2.

5. After last wash, slides were quickly transferred to empty falcon tubes and centrifuged dry for ~3 min at 1 000 rpm.

3.9.3 Scanning, normalization and annotation of data

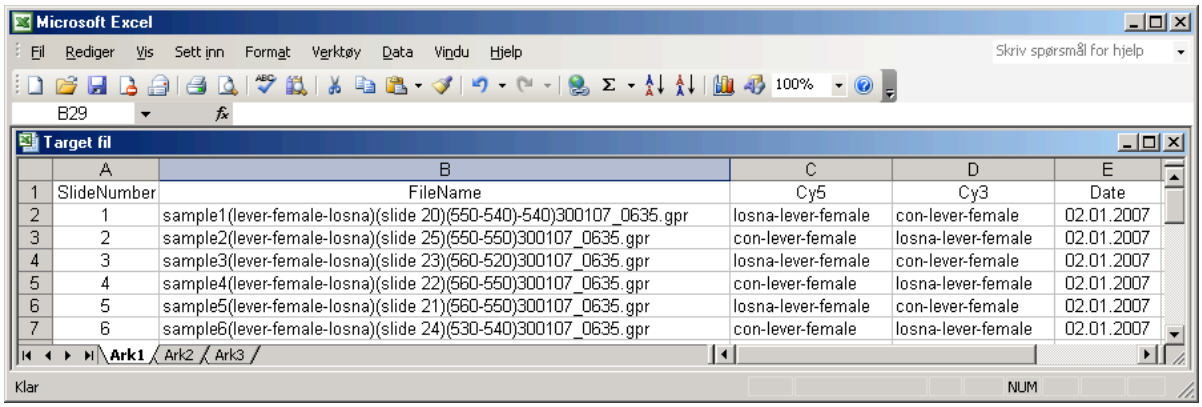
Scanning

Slides were scanned with the GenePix 4000B machine (Molecular Devises, California, USA), which emits light at wavelengths 532 nm (for Cy3 detection) and 635 nm (for

Cy5 detection). The light is converted to an electrical signal and these signals are displayed as images and given numerical values by the GenePix Pro software. The raw data were manually refined to maximize the intensities of the colours, without getting saturated (white) spots, and to balance the foreground and background intensities. Any bad spots were flagged. An array list assigning each spots identity was aligned to the images and the software computed data lists including gene identities, intensity ratios and spot intensities.

Normalization

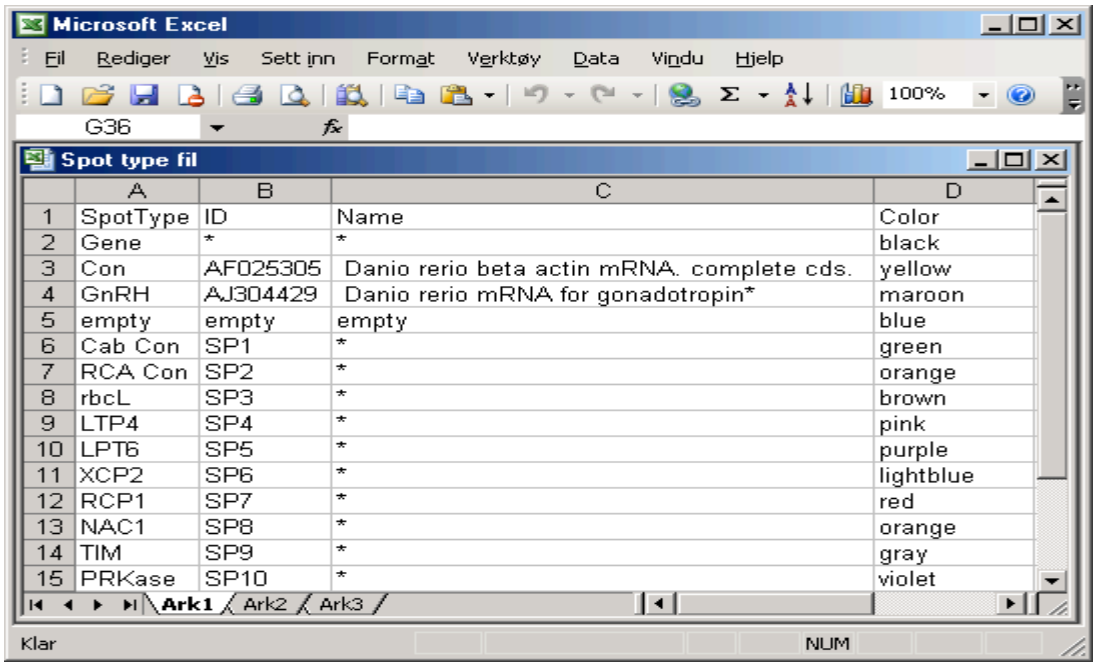
The data computed by the GenePix pro software was exported into the statistical program R with the limmaGUI package loaded. limmaGUI is a Graphical User Interface for the limma (Linear Models for MicroArray data) package. It uses statistical techniques to normalize microarray data, calculate diagnostic plots and to find differently expressed genes, and computes M and A values which are log transformations of the ratio of the colour intensities ($\log_2 R/G$) and the total intensity of the colours in each spot ($1/2\log_2(R*G)$) respectively (R is red and G is green), from the raw data. To identify differently expressed genes, the program needs to have access to a GAL (GenePix Array List) file, a RNA Target file and optionally a Spot Type file. The GAL file was the GenePix Array List that was used to determine each spots identity, while the target and spot type files had to be made manually. The RNA target file was a list of the hybridizations that include the slide number, the name of the hybridization files, and overview over which sample that is labelled green or red and the date of the scanning. It can be made in excel but had to be saved as a tabulator delimited text file for limmaGUI to be able to use it (Fig. 12).



	A	B	C	D	E
1	SlideNumber	FileName	Cy5	Cy3	Date
2	1	sample1(lever-female-losna)(slide 20)(550-540)-540)300107_0635.gpr	losna-lever-female	con-lever-female	02.01.2007
3	2	sample2(lever-female-losna)(slide 25)(550-550)300107_0635.gpr	con-lever-female	losna-lever-female	02.01.2007
4	3	sample3(lever-female-losna)(slide 23)(560-520)300107_0635.gpr	losna-lever-female	con-lever-female	02.01.2007
5	4	sample4(lever-female-losna)(slide 22)(560-550)300107_0635.gpr	con-lever-female	losna-lever-female	02.01.2007
6	5	sample5(lever-female-losna)(slide 21)(560-550)300107_0635.gpr	losna-lever-female	con-lever-female	02.01.2007
7	6	sample6(lever-female-losna)(slide 24)(530-540)300107_0635.gpr	con-lever-female	losna-lever-female	02.01.2007

Fig. 12: *Example of an RNA target file made for use in the limmaGUI program when the GenePix software is used to scan the hybridization samples. The file indicates which of the control samples and exposed samples that are dyed with the different dyes and the raw foreground and background intensities, which are included in the file names.*

The spot type file is useful in distinguishing between genes and controls in a colour-coded MA plot. Also, the control genes can be excluded from a linear model fit when a spot file is used. The file must be saved in the same tab-delimited text format and contained a spot type, an ID (which spot on the slide the gene is on), name and colour column. The second row of the file indicated that every spot in the array was a gene. Below, certain spots were renamed according to the array list, identifying the controls. Different colours were then chosen for the spots representing the genes and the controls (Fig. 13).



	A	B	C	D
1	SpotType	ID	Name	Color
2	Gene	*	*	black
3	Con	AF025305	Danio rerio beta actin mRNA. complete cds.	yellow
4	GnRH	AJ304429	Danio rerio mRNA for gonadotropin*	maroon
5	empty	empty	empty	blue
6	Cab Con	SP1	*	green
7	RCA Con	SP2	*	orange
8	rbcL	SP3	*	brown
9	LTP4	SP4	*	pink
10	LPT6	SP5	*	purple
11	XCP2	SP6	*	lightblue
12	RCP1	SP7	*	red
13	NAC1	SP8	*	orange
14	TIM	SP9	*	gray
15	PRKase	SP10	*	violet

Fig. 13: *Example of a Spot Type file made for use in the limmaGUI program when the GenePix software is used. The spot type column identifies all the genes and controls printed on the slides while the IDs column identifies which spot on the array they represent (found from the GAL file).*

When identifying differently expressed genes, the limmaGUI program gives the option of normalizing the data so that non-biological effects are taken into account. To account for any differences between the spots on an array, data were normalized using a print-tip group LOWESS, which fits a robust local regression to the relation between the M and A values. These fitted values are then subtracted from the original M values to compute the normalized data. Also, to account for the differences in distribution of the M values from array to array, the data were normalized between arrays through a scaling of the M values to give each array the same median absolute deviation. The normalized data were given an adjusted P-value according to the FDR method and exported to excel. Here, all data with an M-value between 0,4 and -0,4 were removed to get more reliable results and eliminating genes differently expressed less than 1.3 fold, together with data having an adjusted P-value above 0,1 leaving only genes with a small enough original P-value that the FDR was only 10 %. The remaining genes

were then sorted as up-regulated (positive M value) or down-regulated (negative M value).

Gene annotation

Finding the gene expression pattern in each organ gives an impression of how differently the organs are affected, but to be able to tell how they are affected one have to know the function of the genes, and different gene annotation databases and tools are available on-line to perform this task. Because annotation is of such great importance it is included in the results together with the differently expressed gene patterns.

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

3.10 Statistics

All data obtained from the sex determination, 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 (the fish were sampled on different days between 161 and 170dpf). The means were compared with the Student's t-test to evaluate significant differences (P-value ≤ 0.05) between the control and exposed fish. The survival data were analyzed using a survival analysis.

All statistical analyses were performed using the JMP 6.0 software from SAS (SAS Institute, Cary, NC, USA), except when identifying the differently expressed genes

where the statistical software R version 2.3.1 was used (http://www.R-project.org/) (R foundation for statistical computing 2004) with the limmaGUI package of Bioconductor (http://www.bioconductor.org/).

4. Results

In this study we have looked at the effects of real life mixtures of POPs isolated from two freshwater systems in Norway, Lake Mjøsa and Lake Losna on two generations of zebrafish by evaluating demographic variables in addition to using traditional toxicological assays and modern microarray technology.

4.1 Exposure to mixtures containing environmental levels of POPs

Zebrafish were indirectly exposed to mixtures of POPs through their diet for six months. Artemia nauplii were incubated with the extracts from the Mjøsa and Losna lakes during hatching and fed to the fish four times a day. The food was exposed to three different dose levels of the Mjøsa mixture and one dose of the Losna mixture while one batch received acetone as the solvent control. The artemia fed to the EE2 group were not treated at all (see M & M).

4.2 Levels of POPs measured

4.2.1 Pilot study

The pilot study aimed at confirming natural levels of pollutant mixtures in the recipient zebrafish, and fish were sampled after 10, 20 and 31 days of exposure and analyzed for BDE-47 and PCB-153 (Table 4). Fish exposed to the Losna mixture contained higher concentrations of PCB-153 than BDE-47 (668 ng/g vs. 158 ng/g (l/w) respectively, after 31 days), while the opposite was the case for fish exposed to the Mjøsa mixture, (393 ng/g vs. 496 ng/g and 552 ng/g vs. 2 927 ng/g for the low and high dose groups respectively). This was what we expected from the levels measured in burbot (see Table 3). After one month the control fish contained approximately the same amounts of both toxins as the fish treated with Losna mixture for ten days.

	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

Table 4: *Concentrations 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 and fish exposed to Losna mixture and two doses of Mjøsa mixture after 10, 20 and 31 days during the pilot study.*

The results of the pilot study gave us information about which concentrations we were to expose the artemia with to get the wanted, environmental relevant levels in the zebrafish after long term treatment. We also decided to treat the oestrogen exposed fish separately because of risk of contamination to the other groups, and this also allowed for water exposure which is a well described method of exposure.

4.2.2 Levels of POPs in Artemia nauplii fed to the fish

When the fish were sexually mature, artemia fed to the different groups were sampled and analyzed for PBDEs and OCs (Table 5 and 6). Artemia that were fed to the

Control group contained only 19,8 ng/g lipid weight of BDE-47, while those given to the Losna group contained 30,4 ng/g. Artemia given to Mjøsa Low, Mjøsa Medium and Mjøsa High groups contained 195 ng/g, 653 ng/g and 3 827 ng/g of BDE-47 respectively (Table 5).

Of the PBDEs, BDE-47 was the congener with the highest measured levels in all five artemia batches. Also, BDE-99 and to some extent BDE-100 accumulated in the mixture treated artemia, and HBCD in the Mjøsa high treated artemia (904 ng/g).

In Mjøsa high treated artemia, the measured concentrations of brominated compounds were BDE-47 > HBCD > BDE-99 > BDE-100 > BDE-154, BDE-153 > BDE-28, while they for Mjøsa medium, low and Losna treated artemia were BDE-47 > BDE-99 > BDE-100 > BDE-154, BDE-153 > BDE-28, HBCD.

The artemia given to the Control group had a fat percent of 1,96, while those given to the Losna, Mjøsa High, Mjøsa Medium and Mjøsa Low groups had a fat percent of 2,15, 2,06, 2,12 and 1,57 respectively. The detection limits were between 0,02-0,03 ng/g wet weight and 1,0-2,0 ng/g lipid weight.

	Artamia				
Component	Control	Losna	Mjøsa Low	Mjøsa Medium	Mjøsa High
BDEs					
BDE-28	n.d	n.d	n.d	n.d	48.1
BDE-47	19.8	30.4	195	653	3827
BDE-100	3.88	5.53	31.8	96.7	467
BDE-99	9.29	16.7	69.6	218	975
BDE-154	1.00	1.49	10.8	26.7	110
BDE-153	1.00	1.77	8.78	25.0	108
HBCD	n.d	n.d	n.d	n.d	908
ΣBDEs	35.0	55.9	316	1020	5534

Table 5: *Concentrations [in ng/g lipid weight (l/w)] of PBDEs and HBCD measured in 1 or 2 g of unexposed artemia and artemia after 24 h of exposure to three different doses of Mjøsa mixture and the Losna mixture. Detection limit was 1,0-2,0 ng/g l/w.*

The concentrations of the OCs HCB, HCHs, Chlordanes, DDTs and PCBs were also measured and none of them were detected in the artemia treated with acetone and fed to the Control group. HCHs and Chlordanes were only detected in the Losna exposed artemia, and only DDE, PCB-101, PCB-138 and PCB-153 accumulated to higher levels in the Mjøsa exposed artemia, while DDE, PCB-138 and PCB-153 accumulated to higher levels in artemia exposed to the Losna mixture (Table 6).

	Artemia				
Component	Control	Losna	Mjøsa Low	Mjøsa Medium	Mjøsa High
	w/w	l/w	l/w	l/w	l/w
HCB	n.d	18.2	14.9	14.8	25.0
HCHs					
α-HCH	n.d	1.40	n.d	n.d	n.d
β-HCH	n.d	1.60	n.d	n.d	n.d
γ-HCH	n.d	1.30	n.d	n.d	n.d
Chlordanes					
oxy-cholrdane	n.d	0.60	n.d	n.d	n.d
Cis-chlordane	n.d	2.00	n.d	n.d	n.d
trans-nonachlor	n.d	7.00	n.d	n.d	n.d
DDTs					
pp-DDE	n.d	27.7	28.5	58.7	431
pp-DDD	n.d	26.3	21.1	23.7	73.1
pp-DDT	n.d	13.5	9.30	23.7	177
ΣDDTs		67.5	58.9	106	681
PCBs					
PCB-28	n.d	8.79	n.d	n.d	38.5
PCB-52	n.d	5.67	n.d	n.d	n.d
PCB-47	n.d	n.d	n.d	n.d	n.d
PCB-74	n.d	1.91	n.d	n.d	n.d
PCB-66	n.d	3.55	n.d	n.d	n.d
PCB-101	n.d	14.2	35.0	54.5	192
PCB-99	n.d	6.60	15.0	22.0	76.9
PCB-110	n.d	15.5	5.68	15.0	112
PCB-151	n.d	1.63	n.d	n.d	n.d
PCB-149	n.d	8.27	n.d	n.d	n.d
PCB-118	n.d	11.0	11.6	16.7	104
PCB-153	n.d	35.5	33.8	56.3	254
PCB-105	n.d	4.18	3.65	6.17	38.5
PCB-141	n.d	2.41	n.d	n.d	n.d
PCB-137	n.d	3.05	n.d	n.d	n.d
PCB-138	n.d	20.8	16.6	31.3	212
PCB-187	n.d	2.84	2.84	4.00	n.d
PCB-183	n.d	1.84	n.d	n.d	n.d
PCB-128	n.d	5.89	3.38	5.00	46.2
PCB-156	n.d	2.91	n.d	n.d	19.2
PCB-180	n.d	1.49	n.d	n.d	19.2
PCB-170	n.d	9.01	8.65	15.0	61.5
PCB-196	n.d	2.62	3.65	5.00	26.9
PCB-189	n.d	n.d	n.d	n.d	n.d

PCB-194	n.d	0.71	n.d	n.d	n.d
PCB-206	n.d	n.d	n.d	n.d	n.d
ΣPCBs		170	140	231	1200
ΣPCB7		97.5	97.0	159	819

Table 6: *Concentrations (in ng/g) of organochlorines measured in 1 or 2 g of unexposed artemia and artemia after 24 h of exposure to three different doses of Mjøsa mixture and the Losna mixture. Levels from solvent exposed artemia (Control) are given in wet weight (w/w) while levels from mixture exposed artemia are given in lipid weight (l/w). Detection limits were 0,02-0,03 ng/g w/w and 1,0-2,0 ng/g l/w.*

4.2.3 Levels of POPs measured in the exposed fish

When the zebrafish were sexually mature, fish were sampled and analyzed for PBDEs and OCs (Table 7 and 8). Table 7 lists the mean levels of brominated POPs measured in the fish, and since the differences between sexes were small, only the levels in females are shown. The Control group contained only background levels of BDE-47, 51,8 ng/g lipid weight, while the Losna group contained about the same, 93,6 ng/g. The Mjøsa Low, Medium and High exposed groups contained 302 ng/g, 1 093 ng/g and 6 599 ng/g lipid weight of BDE-47 respectively. The mixture treated groups also contained elevated levels of BDE-100 and HBCD (Table 7).

The lipid percent of the control fish was 12,4, Losna fish 11,5, Mjøsa low fish 14,5, Mjøsa medium fish 10,9, Mjøsa high fish 11,5 and EE2 fish 7,09.

In all mixture treated fish, the trend of the measured concentrations of brominated compounds were BDE-47 > BDE-100, HBCD > BDE-28, BDE-154, BDE-153 > BDE-99. Detection limit was between 1,0-2,0 ng/g lipid weight.

	Zebrafish					
Component	Control	Losna	Mjøsa Low	Mjøsa Medium	Mjøsa High	EE2
BDEs						
BDE-28	3.56	3.74	9.35	23.8	111	4.07
BDE-47	51.8	93.6	302	1093	6599	64.4
BDE-100	6.89	11.5	29.8	110	633	10.4
BDE-99	0.93	1.01	1.62	7.35	56.5	n.d
BDE-154	2.09	3.09	6.54	21.7	132	3.25
BDE-153	1.62	2.07	6.82	20.8	119	2.42
HBCD	20.1	19.0	45.7	88.6	327	25.7
ΣBDEs	66.9	115	356	1277	7651	84.5

Table 7: *Concentrations in pooled samples [3 fish pooled; in ng/g lipid weight (l/w)] of PBDEs and HBCD in female fish after 160 days of exposure to three different doses of the Mjøsa mixture, the Losna mixture, and ethinylestradiol at 10 ng/L.*

Levels of the different OCs HCB, HCHs, Chlordanes, DDTs and PCBs were also measured in zebrafish (Table 8), and only low concentrations were detected in the control fish, i.e. fish fed solvent exposed artemia. HCHs were not detected in any of the groups and only low levels of chlordanes were found.

Of the DDTs, only DDE accumulated to higher levels in all mixture exposed fish, while DDD was the most abundant metabolite in the Control and POPs untreated EE2 groups. Also, fish exposed to the Losna mixture contained more of both DDT and its metabolites, HCB and PCBs than the Mjøsa Low exposed fish. PCB-138 and PCB-154 were the only two PCB congeners that accumulated in fish exposed to the Losna and Mjøsa mixtures.

	Zebrafish					
Component	Control	Losna	Mjøsa Low	Mjøsa Medium	Mjøsa High	EE2
HCB	11.4	12.3	9.72	10.5	15.8	14.2
HCHs						
α-HCH	n.d	n.d	n.d	n.d	n.d	n.d
β-HCH	n.d	n.d	n.d	n.d	n.d	n.d
γ-HCH	n.d	n.d	n.d	n.d	n.d	n.d
Chlordanes						
oxy-cholrdane	0.81	1.28	0.55	0.69	n.d	1.00
Cis-chlordane	1.69	1.89	1.29	1.51	3.04	2.32
trans-nonachlor	7.02	8.58	5.73	8.19	16.8	12.8
DDTs						
pp-DDE	14.1	48.2	27.1	72.3	551	21.2
pp-DDD	21.7	23.9	15.3	16.1	37.2	27.8
pp-DDT	2.10	6.35	3.32	6.68	36.1	1.86
ΣDDTs	37.9	78.4	45.8	95.1	624	50.9
PCBs						
PCB-28	4.63	6.10	3.77	11.1	16.5	5.16
PCB-52	4.89	5.52	3.97	4.22	13.6	5.46
PCB-47	1.19	1.87	0.87	1.56	5.75	n.d
PCB-74	1.16	3.34	1.41	2.72	16.9	1.55
PCB-66	1.71	4.59	1.73	3.70	24.5	1.86
PCB-101	11.2	16.4	11.1	18.4	68.6	18.7
PCB-99	3.68	7.94	4.56	9.21	52.1	6.08
PCB-110	14.7	21.7	14.4	24.6	118	23.1
PCB-151	3.14	5.10	3.07	3.95	17.3	5.36
PCB-149	9.71	10.4	8.24	11.2	25.7	16.0
PCB-118	4.80	16.4	8.06	19.2	128	7.73
PCB-153	17.2	45.6	24.5	61.8	316	28.1
PCB-105	2.09	5.40	2.93	7.63	51.6	3.61

PCB-141	2.33	3.57	2.18	3.36	14.9	3.25
PCB-137	1.28	1.25	1.14	1.54	7.67	14.0
PCB-138	11.7	29.8	16.2	39.6	225	17.9
PCB-187	3.49	4.27	2.90	4.09	13.1	5.31
PCB-183	2.11	3.49	2.14	3.51	18.4	3.35
PCB-128	1.57	4.10	2.46	6.23	40.9	2.11
PCB-156	n.d	4.12	2.19	4.64	28.6	2.63
PCB-180	n.d	1.29	0.98	2.13	5.27	1.91
PCB-170	6.89	13.0	7.47	15.6	82.6	11.1
PCB-196	2.23	4.23	2.67	5.19	29.7	3.25
PCB-189	n.d	0.80	0.46	0.52	2.08	n.d
PCB-194	1.12	0.95	1.08	1.58	5.91	2.17
PCB-206	n.d	0.47	0.41	0.69	2.24	n.d
ΣPCBs	113	222	131	268	1332	190
ΣPCB7	54.4	120	68.5	156	774	85.0

Table 8: *Concentrations in pooled samples [3 fish pooled; in ng/g lipid weight (l/w)] of organochlorines in female fish after 160 days of exposure to three different doses of the Mjøsa mixture, the Losna mixture, and EE2 at 10 ng/L. Detection limit was 1,0-2,0 ng/g l/w.*

4.2.4 Levels of POPs measured in burbot, artemia and zebrafish

When analyzing livers from burbot caught in Lake Mjøsa, elevated levels of DDE, PCB-138 and PCB-153 were found in addition to the high levels of PBDEs (Table 3). Comparing the data of POPs measured in artemia (Table 5 and 6) and in zebrafish (Table 7 and 8) reveals a correlation between levels in liver oils of burbots, in exposed live food and in zebrafish fed with exposed artemia (Fig. 14). The only exception was BDE-99 in zebrafish, which did not accumulate at all.

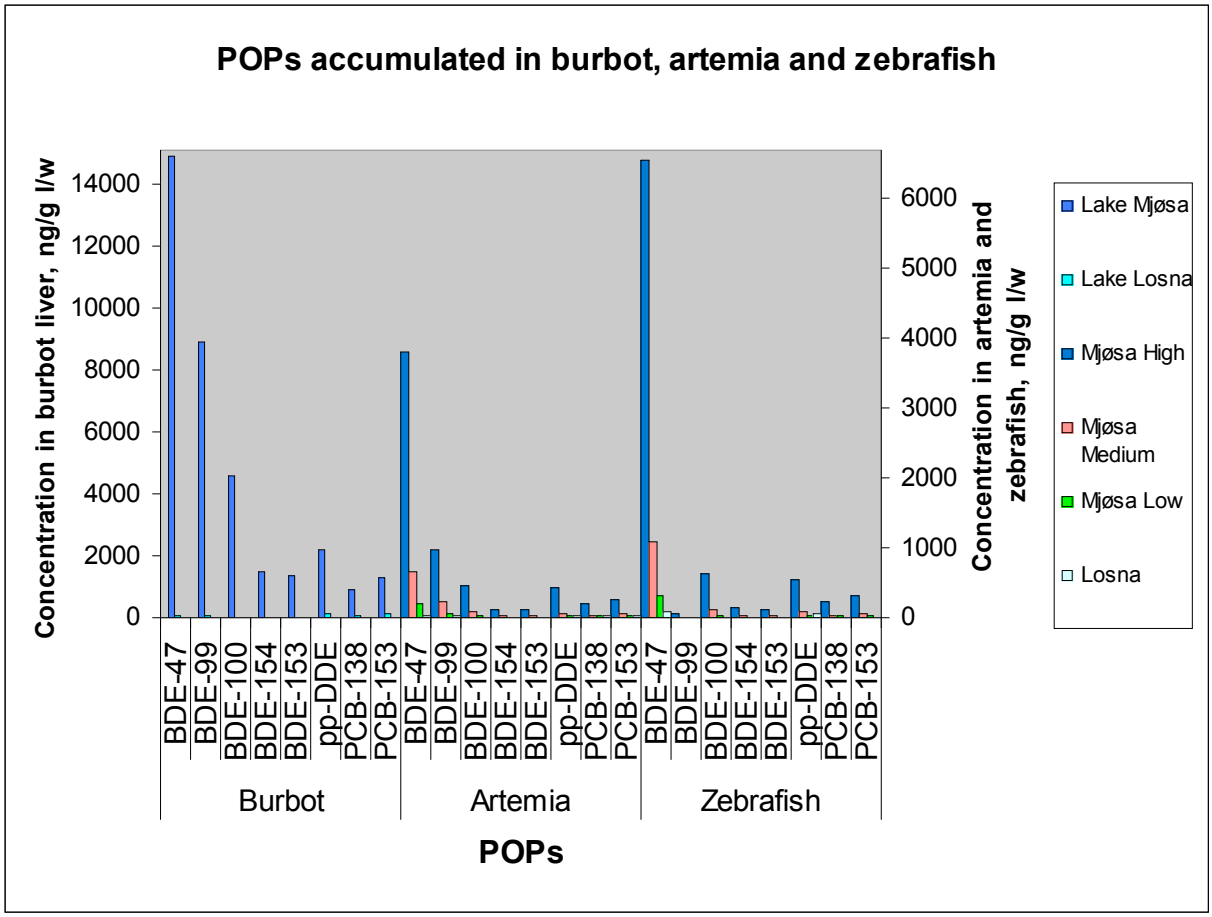


Fig. 14: *Comparison of the accumulation of toxins in livers of burbots caught in Lake Mjøsa and Lake Losna, and in Artemia nauplii after 24 hours of exposure and zebrafish after 160 days of exposure to thee different dose levels of the Mjøsa mixture, the Losna mixture and solvent as control. Concentrations in burbot livers are given on the left y-axis, while concentrations in artemia and zebrafish are given on the right y-axis, and all are measured in ng/g lipid weight (l/w). Control artemia and zebrafish exposed to acetone showed only low levels of any of the toxins. Mixtures were obtained from the burbot livers and those caught in Lake Mjøsa contained high levels of PBDEs, in addition to DDTs and PCBs, while those caught in Lake Losna contained low levels of all three toxins.*

4.3 Demographic variables

Demographic variables such as survival, growth and sex ratio were monitored throughout the experiment or assessed when sampling of tissue took place.

4.3.1 Survival of the fish

F_0 generation

A high mortality rate was observed in all groups between 10 and 20dpf, but the toxin-treated groups showed significantly higher mortality rates than controls. After this rapid decline in survival, all groups stabilised except the EE2 group, which showed a gradual decline in survival throughout the experiment (Fig. 15). At 40dpf, the EE2 and Control groups had significantly better survival rates than the other groups, while at 160dpf the oestrogen treated group was nearly at the same level as the real life mixture exposed groups (Fig. 15). The Control group had the best survival rate at 160dpf with 76.3 %. Of the mixture treated group, Losna and Mjøsa high exposed fish had the highest mortality, followed by Mjøsa medium and Mjøsa low treated fish.

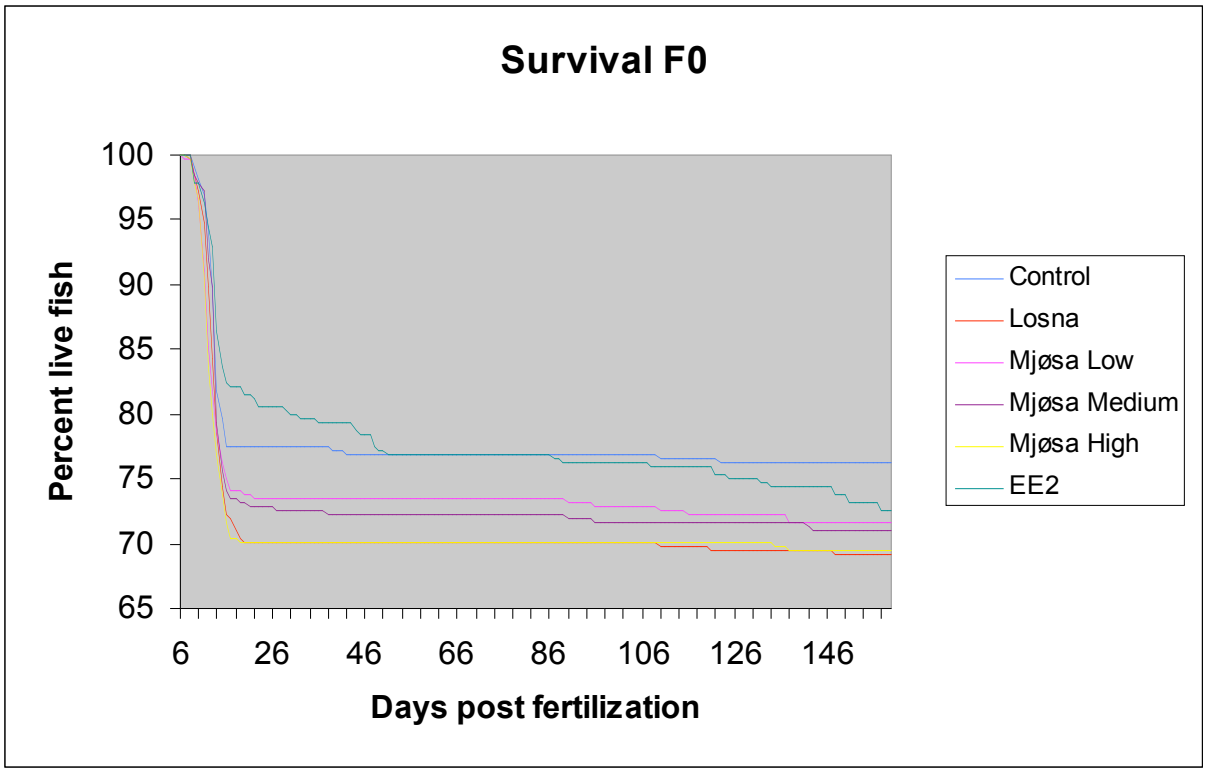


Fig. 15: *Mean survival curves for the F_0 generation after long term exposure to two different real life mixtures from the Mjøsa and Losna area, at different doses, and oestrogen at 10 ng/L. Each line represents the mean of five replicates each containing 65 larvae on 6dpf.*

F_1 generation, before exposure (0-6dpf)

The survival rate of the second generation was monitored the first six days after spawning (Fig. 16), and already after 24 hours, eggs from control parents showed significantly better survival than those from Losna and Mjøsa treated parents (Fig. 16). All three groups showed a higher mortality rate the first two days after spawning which decreased between day 2 and 6, except in the Losna group which showed an increase in mortality rate from day 5 to day 6 (Fig. 16).

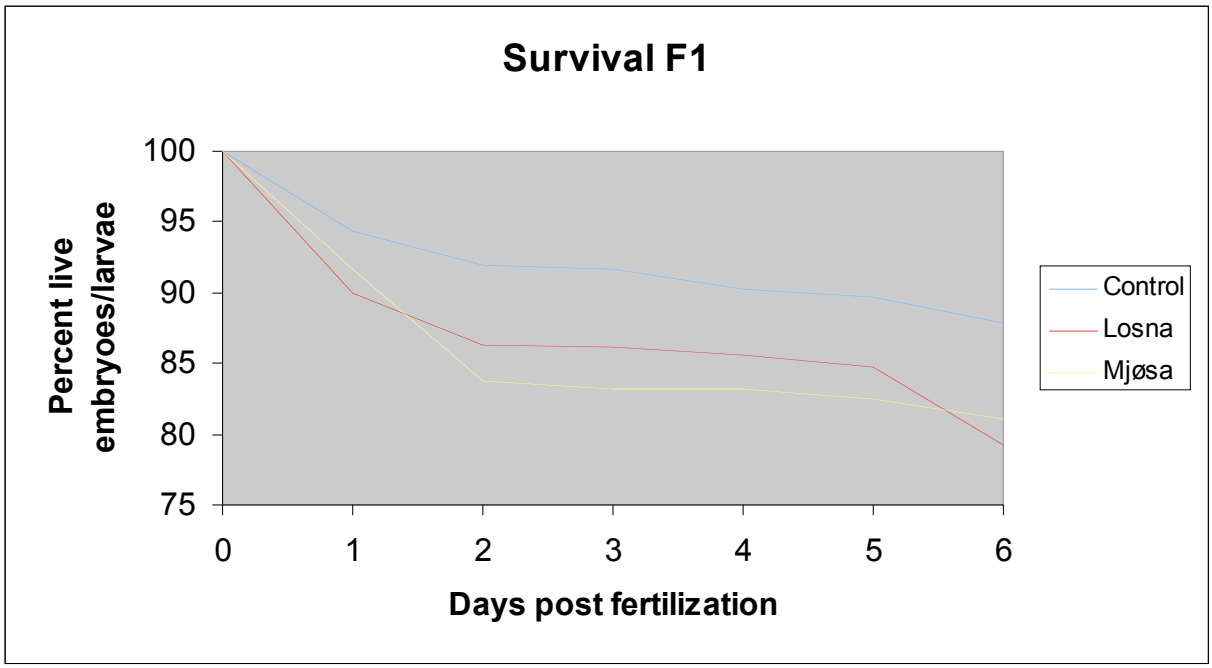


Fig. 16: *Mean survival curves for the F_1 generation before start of exposure. The parent generation was exposed to real life mixtures from Lake Losna or Lake Mjøsa (the high dose). Each line represents the mean of five replicates, each containing 200 embryos at day 0.*

F_1 generation, after start of exposure (6- dpf)

A high mortality rate was observed between 10 and 16dpf in all groups but after 16dpf the mortality rate decreased and stabilised at around 20dpf (Fig. 17). At 62dpf, the Losna-Losna group had the best survival rate of 66,8 %, while the Mjøsa-Control, Control-Control and Control-Losna groups followed closely with 66,5 %, 64,8 % and 64,5 % survival respectively. The Mjøsa-Mjøsa, Losna-Control and Control-Mjøsa

groups showed a significantly higher mortality rate than the other groups (Fig. 17) with 59,8 %, 54 % and 43,8 % survival respectively at day 62.

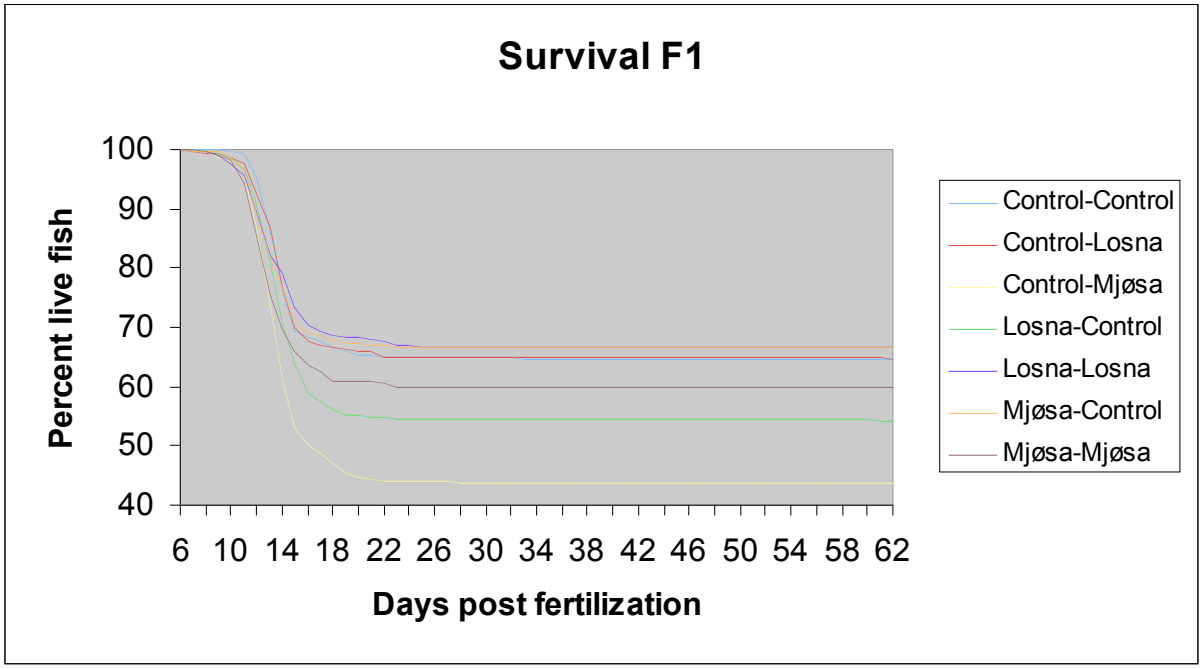


Fig. 17: *Mean survival curves for the F_1 generation after two months of exposure to mixtures originating from burbots caught in Lake Mjøsa and Lake Losna. The first part of the group names refers to what treatment the parents were exposed to, while the last part indicates what treatment they themselves are getting. Each line represents the mean of five replicates, each containing 85 larvae on 6dpf.*

The mortality was higher in F_1 as compared to F_0 for all groups, and while the survival of the Control group was 76,3 % on 160dpf in the F_0 generation it was only 64,8 % on day 62 in the F_1 generation. The lowest survival observed in F_0 was the Losna group with 69,2 %, while the lowest observed in F_1 was 43,8 % in the Control-Mjøsa group.

4.3.2 Sex ratio

The sex of the F_0 fish was determined when they reached sexual maturity, and the male/female sex ratio was significantly higher in the mixture treated groups together than in controls (Fig. 18). The Losna group had the lowest mean (n = 32) proportion of females, (39,6 %), while the Mjøsa Low, Mjøsa High, Mjøsa Medium and Control groups had 40,7 %, 41,4 %, 42,0 % and 45,8 % females respectively (Mjøsa High,

Mjøsa Medium and Mjøsa Low; n = 35, Control; n = 56). Only the Losna group differed significantly from the Control group in pair wise comparisons, and the Mjøsa High group was borderline significantly different from controls (P = 0,11) Also, the three Mjøsa groups were significant different from the control when combined. The variation in sex ratio between replicates was large (Fig. 18). The oestrogen exposed group had only phenotypic females.

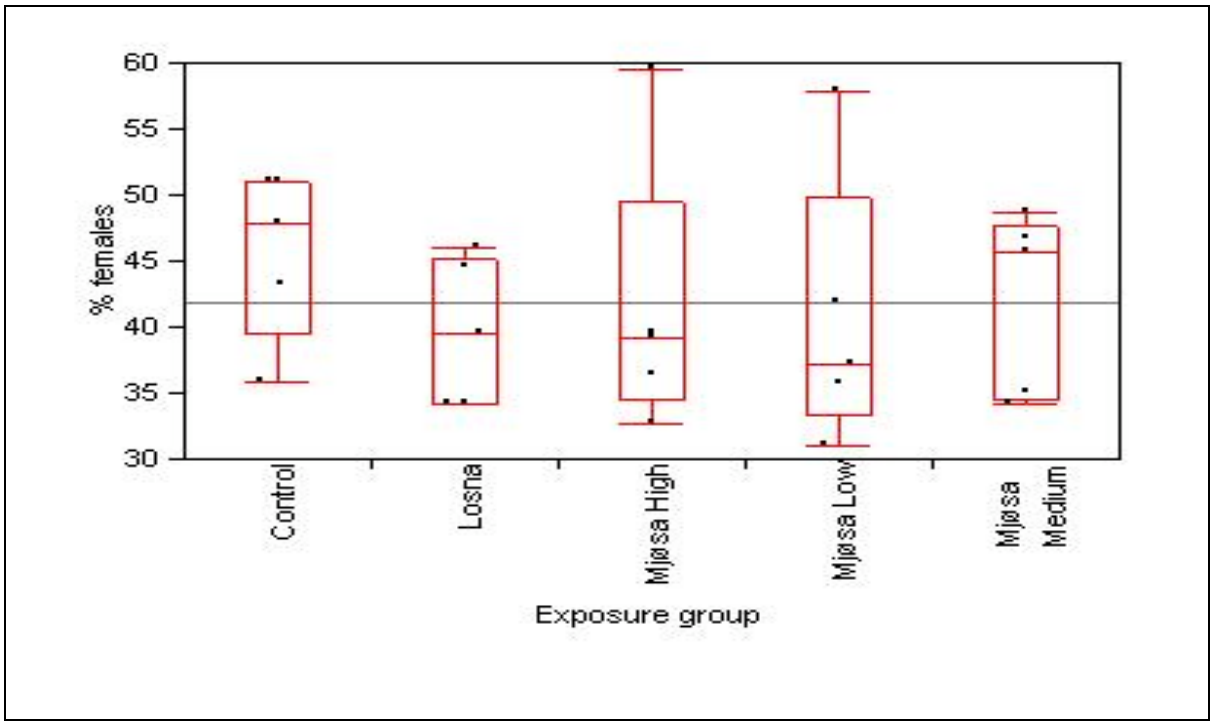


Fig. 18: *Distribution of phenotypic F_0 females in the five replicates of the control group and groups exposed to real life mixtures of POPs extracted from the Mjøsa and Losna area. In comparison, 100 % females were observed in the EE2 group, at 10 ng/L (not depicted here). The horizontal lines of the boxes indicate the smallest observation, the lower quartile (Q1), the median, the upper quartile (Q3) and the largest observation, while the black dots are the actual observations.*

4.3.3 Body mass and length of sampled fish

When the F_0 fish were sexually mature, all fish sampled were weighed and measured, and the mean body mass of the fish sampled on 161-170dpf ranged from 380 to 653

mg (Fig. 19), (Control: females n = 50, males n = 65, Losna: females/males n = 60, Mjøsa High, Medium and Low: females n = 55, males n = 65, EE2: females n = 63). The female oestrogen treated fish weighed significantly less than all other groups, while, when correcting for gender, exposure to toxins had a significant positive effect on body mass. However, there was no significant difference in mean body mass between the to mixture types, Mjøsa and Losna. Females had a significant higher body mass than males (Fig. 19).

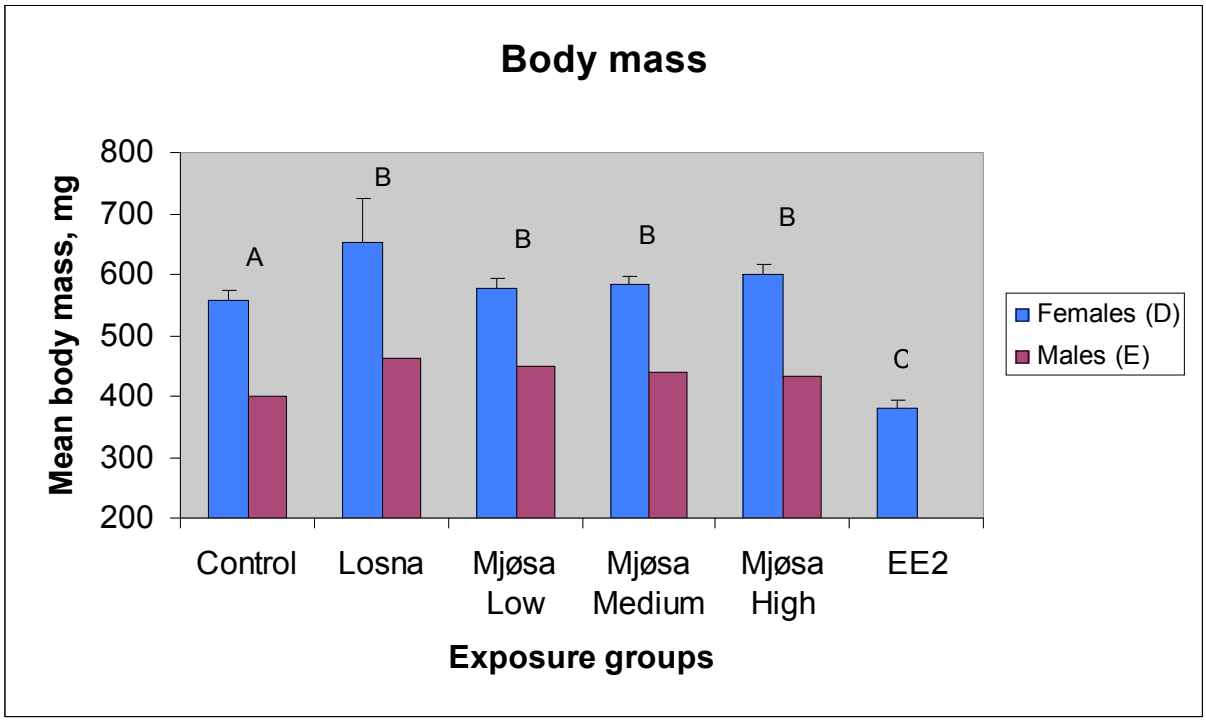


Fig. 19: *Phenotypic, gender specific mean body mass (+SE) on 161-170dpf in untreated controls and in F_0 zebrafish exposed to real life mixtures of POPs extracted from burbot liver in two different locations (Mjøsa and Losna) in a fresh water system in Norway, and oestrogen exposed zebrafish (water exposure of ethinylestradiol; at 10 ng/L). Groups not connected by the same letter are significant different.*

The mean length of the sampled fish varied from 32,8 to 37,2 mm (Control: females n = 50, males n = 65, Losna: females/males n = 60, Mjøsa High, Medium and Low: females n = 55, males n = 65, EE2: females n = 63). The only group significantly different from the Control group was the EE2 exposed, however, when correcting for

gender, the mean body length in the Losna group was significant higher than in the Mjøsa High group.

Table 9 gives an overview over the least square means and standard errors for the body mass and length of the sampled fish.

	Body mass (in mg)		Length (in mm)	
Group	Least Sq Mean	Std Error	Least Sq Mean	Std Error
Control	464.21	23.17	35.86	0.4950
Losna	509.49	23.06	36.36	0.4936
Mjøsa Low	502.58	23.07	36.25	0.4934
Mjøsa Medium	498.77	23.06	36.08	0.4932
Mjøsa High	504.67	23.07	35.81	0.4934
EE2	305.83	24.60	32.22	0.5290
Females	530.38	22.08	35.91	0.4698
Males	398.14	22.12	34.96	0.4707

Table 9: *Least sq means (+SE) for body mass and length of fish exposed to real life mixtures of POPs harvested from the Mjøsa and Losna area and oestrogen, and of the two sexes.*

4.4 Analysis of biomarkers

When the F_0 fish were sexually mature, two male fish from each replicate of all the groups were sampled and analyzed for traditional biomarkers indicating dioxin-like exposure and the presence of xenoestrogens.

4.4.1 EROD activity

The EROD activity was measured in gill arches dissected from the fish, and the means varied from 0,0537 to 0,0692 pmol resorufin/gill arch/min (Control and Mjøsa Low; n = 29, Losna and Mjøsa High; n = 30, Mjøsa Medium and EE2; n = 28) (Fig. 20), and it was significantly lower in the Control group than in all other groups (Fig. 20). Also, the Losna, Mjøsa High and Mjøsa Medium groups showed significantly lower EROD activity than the Mjøsa Low and EE2 groups, which had the highest activities measured.

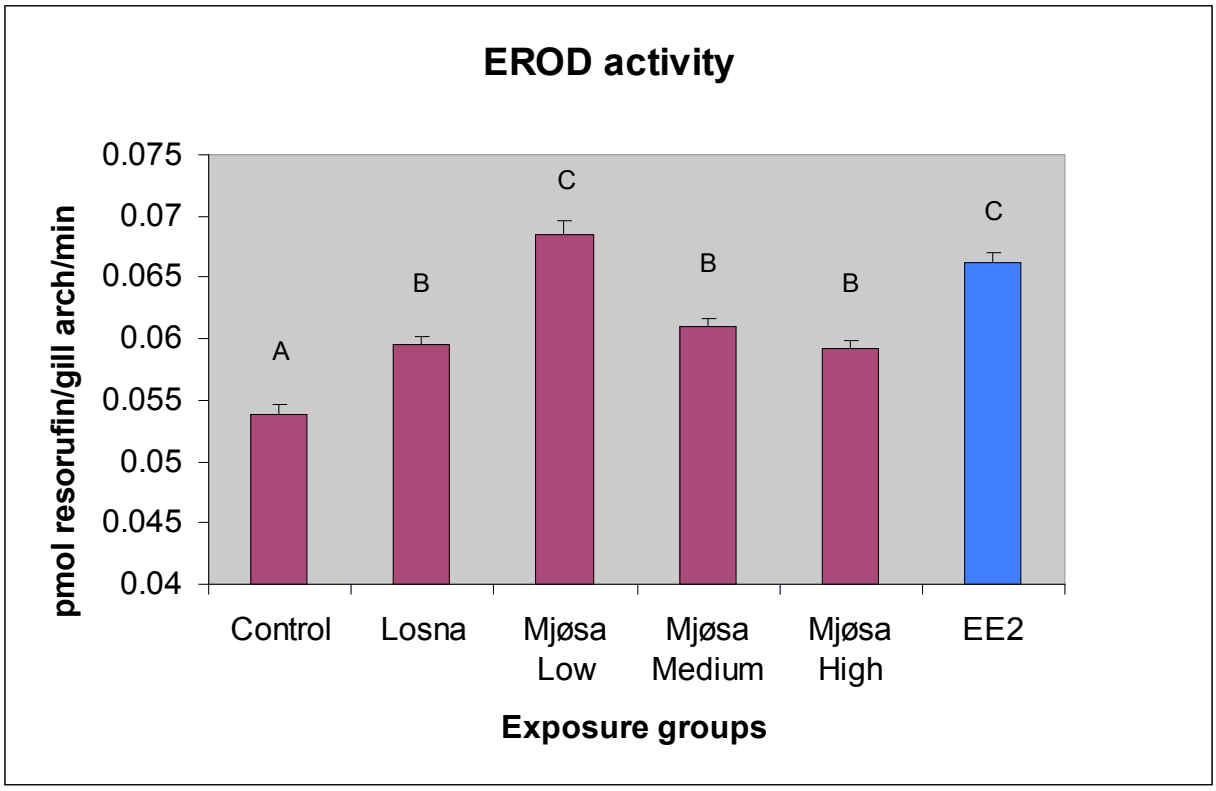


Fig. 20: *Mean concentrations (+SE, Control and Mjøsa Low: n = 29, Losna and Mjøsa High: n = 30, Mjøsa Medium and EE2: n = 28) of resorufin produced in the gills of the fish in each group. EROD activity was measured in males (pink) in all groups except in the oestrogen group, where it was measured in phenotypic females (blue). Levels are expressed in pmol resorufin/gill arch/min. Groups not connected by the same letter are significantly different.*

4.4.2 Vitellogenin

The vitellogenin concentrations were measured in whole body homogenate and the means varied from 9 985 ng/ml in the Control group to 588 600 ng/ml in the EE2 group (Fig 21), (Control; n = 9, Losna, Mjøsa Low, Mjøsa Medium, Mjøsa High and EE2; n = 10). Only the Mjøsa Low and EE2 groups were significantly different from the controls, however, when analyzing the three Mjøsa groups together, the fish treated with the Mjøsa mixture were almost significant different from the Control group (P-value = 0,0942). Vtg was measured in males in all groups except the oestrogen treated group, which only contained phenotypic females.

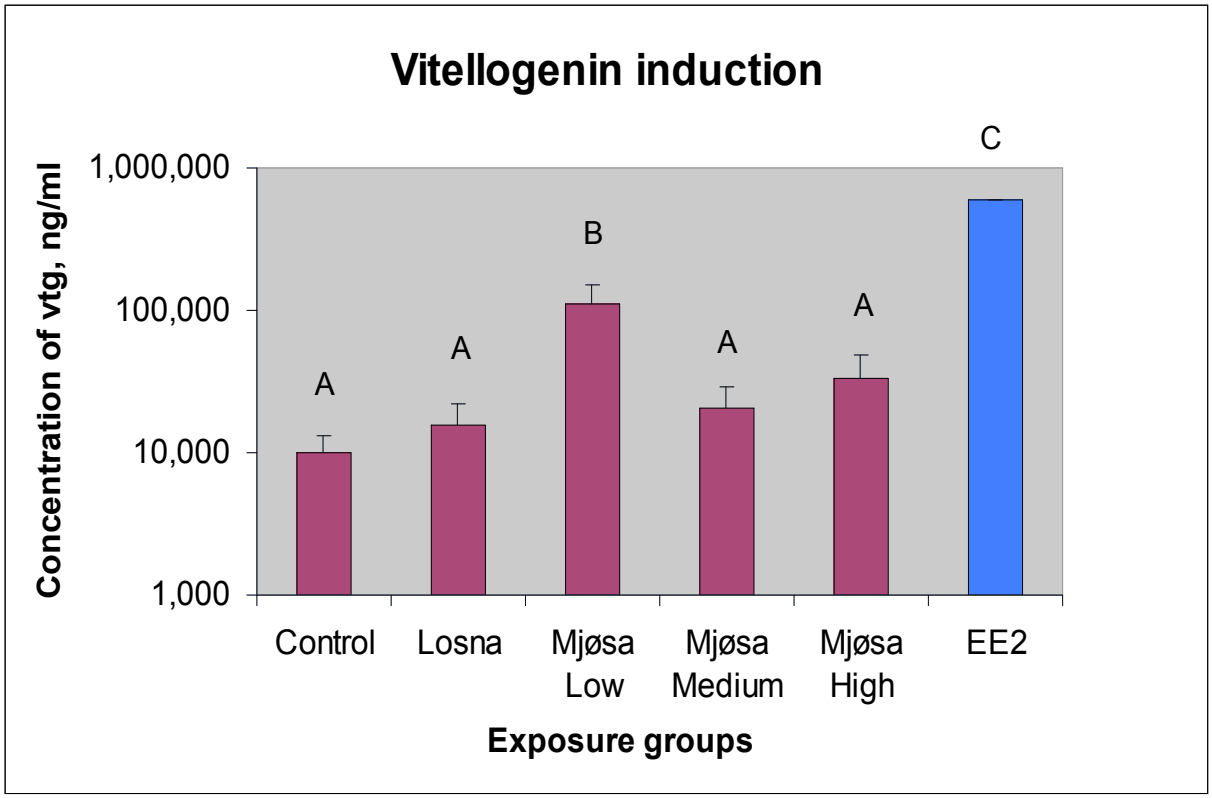


Fig. 21: *Mean measured concentrations of Vtg (in ng/ml, Control; n=9, Losna, Mjøsa High, Mjøsa Low, Mjøsa Medium and EE2; n=10) in whole body homogenate of unexposed fish and zebrafish exposed to the Losna or Mjøsa mixture, or ethinylestradiol at 10 ng/L. Vtg levels were measured in males (pink) in all groups except in the oestrogen group where it was measured in phenotypic females (blue). Groups not connected by the same letter are significant different.*

4.5 Microarray analysis

Six female F_0 fish were sampled from each replicate of all groups after 160dpf and organ specific differences in gene expression patterns were investigated in Losna and Mjøsa high exposed fish compared to the controls using microarray technology with arrays containing zebrafish oligos.

4.5.1 Results of total RNA isolation

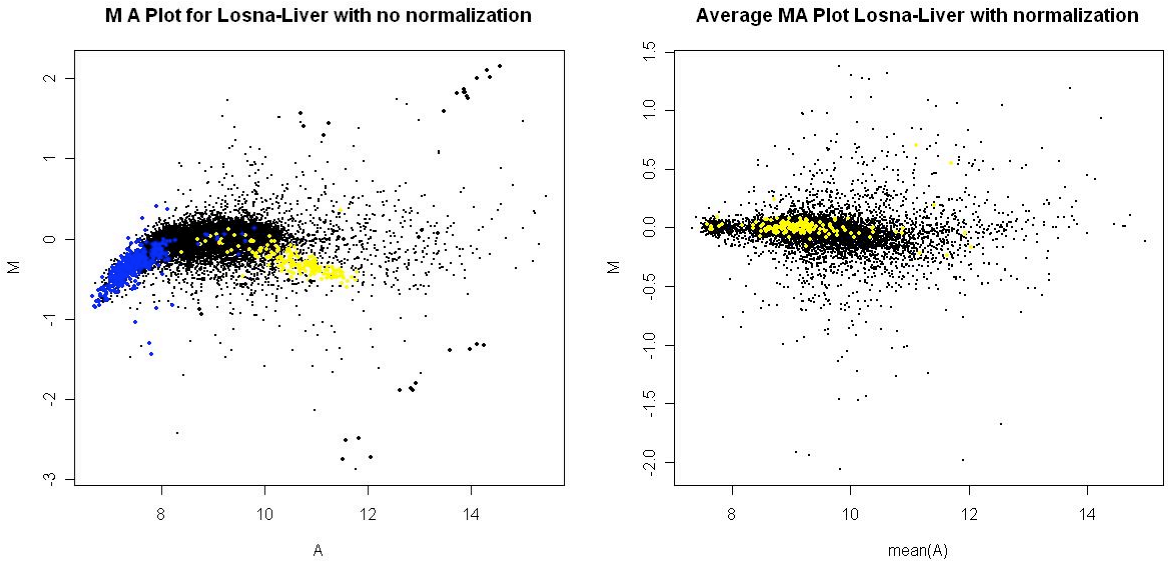
Of the 36 samples (6 pooled ovary and 6 pooled liver samples for each of the Control, Losna and Mjøsa High groups) total RNA was isolated from, all had a concentration

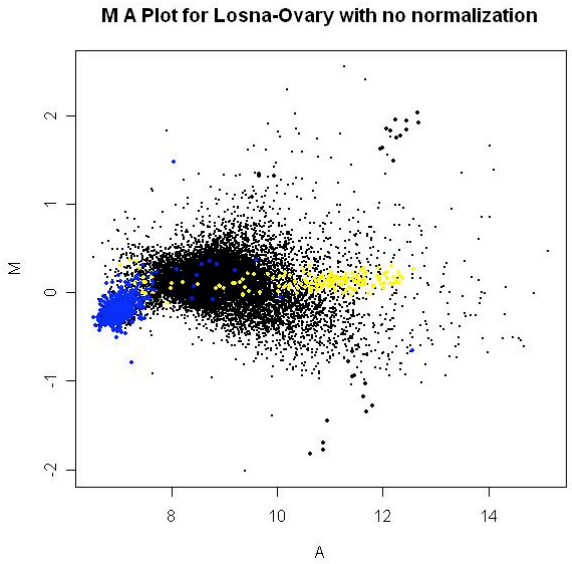
of 700 ng/µl or more when measured after the last isolation step. This was enough RNA to continue with microarray analysis

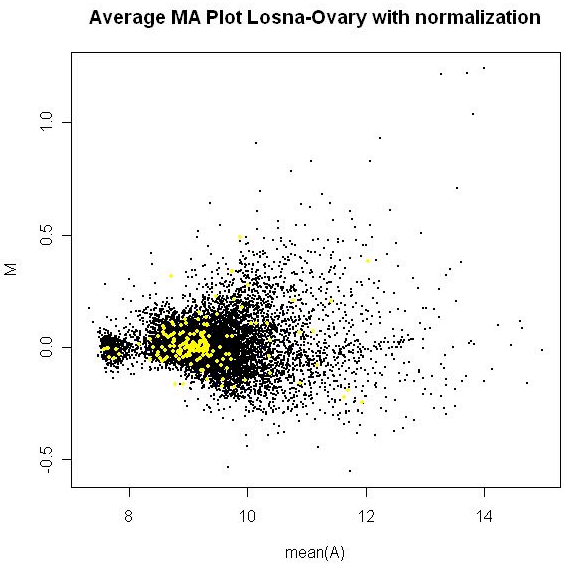
Also, of the 36 samples, total RNA was intact and no degradation observed in any of the samples after measuring with the Bioanalyzer 2100 (Agilent Technologies, California, USA), as assessed by the high RIN numbers observed or through comparison of the electrophoretic profiles of the samples.

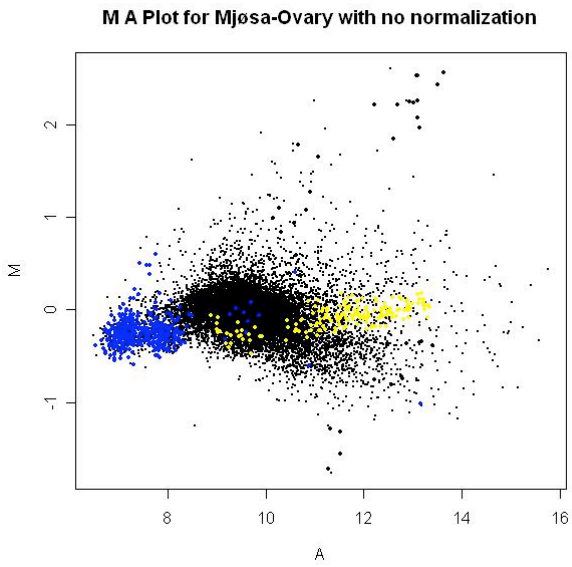
4.5.2 Results of normailzation and differently expressed genes

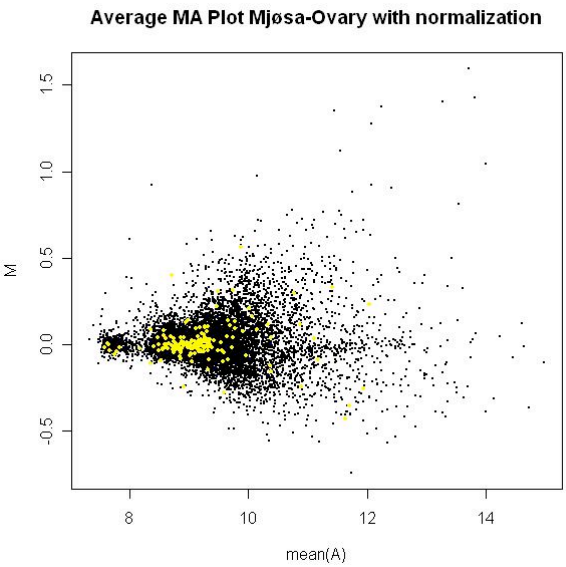
Normalization was performed to account for non-biological effects and examples of pre-normalized data from one slide and results of post-normalized hybridization data of the average of all six slides in each comparison are shown below (Fig. 22). All comparisons are to the controls.

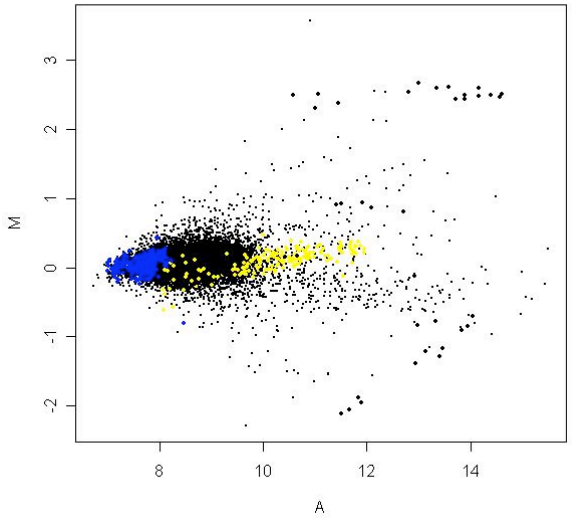



M A Plot for Losna-Ovary with no normalization
M
A
-2
-1
0
1
2
8
10
12
14


Average MA Plot Losna-Ovary with normalization
M
mean(A)
-0.5
0.0
0.5
1.0
8
10
12
14


M A Plot for Mjøsa-Ovary with no normalization
M
A
-1
0
1
2
8
10
12
14
16


Average MA Plot Mjøsa-Ovary with normalization
M
mean(A)
-0.5
0.0
0.5
1.0
1.5
8
10
12
14


M A Plot for Mjøsa-Liver with no normalization
M
A
-2
-1
0
1
2
3
8
10
12
14

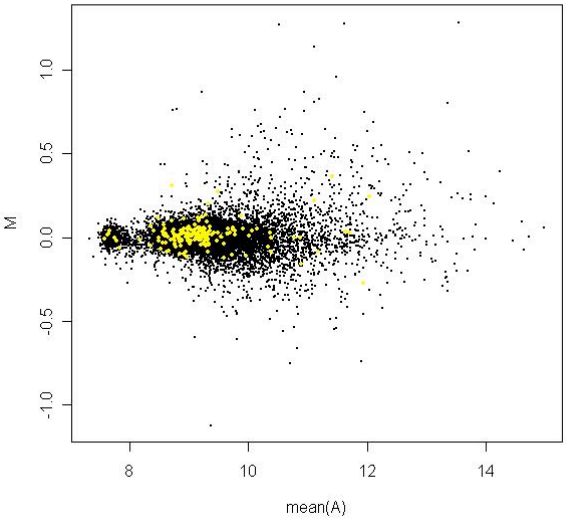

Average MA Plot Mjøsa-Liver with normalization
M
mean(A)
-1.0
-0.5
0.0
0.5
1.0
8
10
12
14

Fig. 22: *MA plots showing examples of pre-normalized data from single slides and post-normalized data from the average of six slides of gene expression patterns of the livers and ovaries after exposure to the Mjøsa and Losna mixture. (M being the log value of the difference in intensity and A being the log value of the total intensity for each spot) After normalization, the data are more straight out and centralized around M = 0. The Spot Type file defines the coloured dots, and the blue spots represent empty spots on the array (genes not expressed in the organ), while the yellow spots represent the housekeeping gene beta actin. Black spots are the mRNAs detected.*

The difference in mRNA concentrations was identified using the limmaGUI program in the statistical software R and exposure resulted in a difference in mRNA levels of 660 genes. Treatment with the Mjøsa mixture resulted in a difference in mRNA levels of 85 genes in livers, where 61 were up-regulated and 24 were down-regulated. In the ovaries, mRNA levels of 210 genes were differently expressed with 177 up-regulated and 33 down-regulated. Exposure to the Losna mixture resulted in a difference in mRNA levels of 288 liver genes, with 116 up-regulated and 172 down-regulated, while 77 genes were differently expressed in the ovaries with 74 being up-regulated and 3 down-regulated (Table 10). The differently regulated mRNA levels identified were all significant with a FDR of 10 % or lower.

4.5.3 Gene annotation

Gene annotation is important to find the function of the differently expressed gene transcripts, and was performed using the on-line program DAVID. Of the 288 differently expressed Losna liver mRNAs, DAVID recognized 213 genes but only 123 had a known function. Similarly, DAVID recognized 59 out of 77 differently expressed Losna gene transcripts in the ovaries and was able to annotate 30 of them. The Mjøsa exposure affected 85 different liver transcripts, of which 63 were recognized by DAVID and 43 annotated, while 168 of the 210 differently regulated ovary transcripts were recognized and 92 annotated (Table 10).

	Losna				Mjøsa			
	Liver		Ovary		Liver		Ovary	
	Up	Down	Up	Down	Up	Down	Up	Down
DAVID annotated	49	74	29	1	27	16	70	22
Unknown function	36	54	28	1	14	6	69	7
Not recognized	31	44	17	1	20	2	38	4
Total	116	172	74	3	61	24	177	33

Table 10: *Overview over differently expressed gene transcripts in livers and ovaries after exposure to the Mjøsa and Losna mixture, and which that were recognized and annotated by the web accessible gene annotation program DAVID. In total, 660 gene transcripts were differently regulated in the livers and ovaries of mixture exposed fish.*

The genes representing the differently regulated mRNA values were, after annotation, divided into functional classes based on the gene ontology number that was linked to their accession number using the DAVID functional annotation chart tool. This is usually done to get an overview over which processes are affected in the cells.

95 of the 123 identified differently expressed Losna liver genes were classified, while 21 of the 59 ovary genes with known function were classified. Of the Mjøsa genes, 27 of the 63 liver genes and 73 of the 168 ovary genes with known identity were classified (Table 11).

	Losna				Mjøsa			
	Liver		Ovary		Liver		Ovary	
	Up	Down	Up	Down	Up	Down	Up	Down
Catalytic activity	17	35					23	9
Cellular macromolecule metabolism	12	11			4	7	12	8
Cellular metabolism	19	31			11	8	25	13
Cellular physiological process	26	42	13	1			35	14
Cellular process	27	44	15	1			38	16
Cellular protein metabolism					4	6	12	8
Establishment of localization	6	16					14	3
Hydrolase activity	7	10	5	1			13	6
Localization	6	16					14	3
Macromolecule metabolism	14	17			6	7	17	10
Metabolism	24	34			14	9	29	13
Nucleic acid binding	4	7	8	0				
Physiological process	31	48	14	1	17	10	40	14
Primary metabolism	20	27			12	8	28	12
Protein metabolism					5	6	13	9
Response to stimulus	4	7			3	3		
Transport	2	16					13	3

Table 11: *Classification of the enriched genes based on gene ontology numbers after exposure to the Mjøsa and Losna mixture. Many genes are classified in more than one group.*

A comparison was also done between the mRNA transcripts differently regulated in each organ to see the individual effects of the two mixtures (Fig. 23). In the ovaries, 50 of the same mRNA transcripts were up-regulated after treatment, corresponding to 67 % of the total number of regulated transcripts in ovaries of Losna treated fish and 28 % of the regulated mRNA transcripts in ovaries of Mjøsa treated fish. In comparison, 100 %, that is all three of the down-regulated mRNA transcripts in ovaries of Losna exposed fish were also found in the ovaries of Mjøsa treated fish, however, here they only accounted for 9 % of the total amount of the down-regulated transcripts. In the liver, 22 of the same transcripts were up-regulated after exposure to the Losna and Mjøsa mixture, corresponding to 19 % and 36 % of the total number of up-regulated liver transcripts respectively, while 9 identical transcripts were down-regulated after Losna and Mjøsa treatment, corresponding to 5 % and 37 % of the total number of down-regulated liver transcripts.

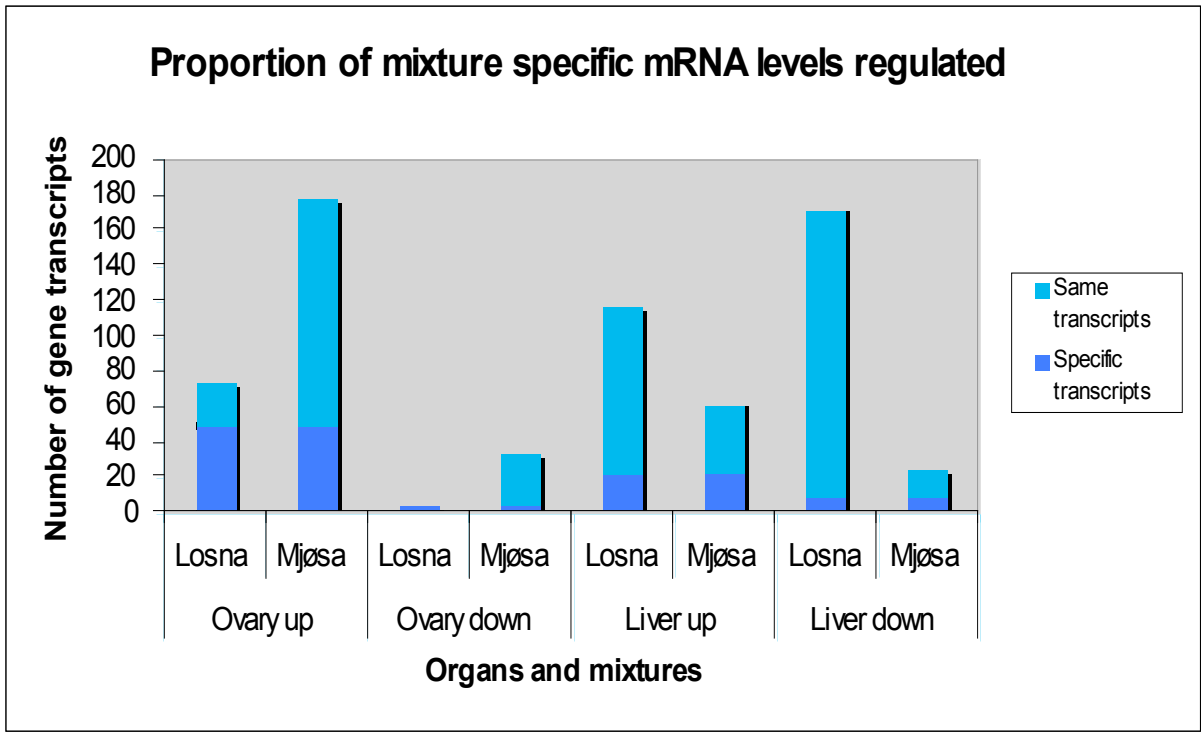


Fig. 23: *Overview over the proportions of regulated mRNA transcripts in the ovaries and livers which were regulated dependently or independently on the type of mixture (Mjøsa or Losna) the fish were exposed to.*

Table 12 gives an overview over differently regulated mRNA transcripts identified after annotation that are of special interest because they are involved in regulation of the endocrine system or in biotransformation or other processes known to be effected by toxin exposure. Gene names coloured red represent up-regulated transcripts while green colour represent down-regulated transcripts. mRNA coding for the egg yolk precursor protein vitellogenin was found up-regulated in livers of fish exposed to the Losna mixture (2,3 fold) as well as in ovaries of both Losna and Mjøsa treated fish (1,9 and 2,6 fold respectively). Also, in livers of Losna treated fish and ovaries of Mjøsa exposed fish a vitellogenin like mRNA transcript was up-regulated 2,1 and 2,5 fold respectively. Other interesting up-regulated liver transcripts in Losna treated fish included metallothionein (1,9 fold), angiotensinogen (1,5 fold) and FK506 binding protein 5 and 11 (1,4 and 1,8 fold) and sterol-C4-methyl oxidase like gene (1,7 fold) which when translated bind physiological and xenobiotic heavy metals, are connected to steroid hormone activity and is linked to cholesterol depletion respectively. Of the

down-regulated transcripts, mRNA coding for the phase I and II biotransformation enzymes CYP3A, sulfotransferase 2 and 6 and glutathione-S-transferase (1,5, 1,3 and 1,3 fold respectively) were found, together with iodothyronine mRNA (2,8 fold) which when translated is involved in thyroid hormone potency by catalyzing the conversion of thyroxine (T_4) to triiodothyronine (T_3) and 17 β-hydroxysteroid dehydrogenase 12A mRNA (1,3 fold) which code for an enzyme involved in the production of E2. In livers of Mjøsa treated fish, mRNA coding for the AhR interacting protein, which when translated is involved in the AhR response pathway, was found 1.5 fold down-regulated. In ovaries of Mjøsa treated fish, CYP19A and FK506 binding protein 5 mRNAs, both involved in steroid hormone activity when translated, were up-regulated in addition to vitellogenin 1,6, 1,4 and 2,6 fold respectively, while mRNA coding for the egg membrane protein zona pellucida was down-regulated 1,3 fold (Table 12).

ID	Gene Name	M-value	Times regulated ($2^{M\text{-value}}$)	FDR adj. P-value
Losna liver				
BF717943	VITELLOGENIN 1	1.191	2.3	0.0556
AI545930	VITELLOGENIN 1, LIKE	1.036	2.1	0.0453
AW184187	METALLOTHIONEIN 2	0.933	1.9	0.0040
BG308728	FK506 BINDING PROTEIN 5	0.459	1.4	0.0237
BI427744	FK506 BINDING PROTEIN 11	0.838	1.8	0.0023
BG727310	ANGIOTENSINOGEN	0.558	1.5	0.0002
AI522669	STEROL-C4-METHYL OXIDASE-LIKE	0.740	1.7	3.85E-08
AY452279	CYTOCHROME P450, FAMILY 3, SUBFAMILY A, POLYPEPTIDE 65	-0.872	-1.8	3.92E-12
BC075996	CYTOSOLIC SULFOTRANSFERASE 6	-0.406	-1.3	1.36E-06
BC047850	CYTOSOLIC SULFOTRANSFERASE 2	-0.601	-1.5	8.93E-04
CF416980	GLUTATHIONE S-TRANSFERASE THETA 1	-0.416	-1.3	1.79E-03
BI671189	DEIODINASE, IODOTHYRONINE, TYPE II	-1.470	-2.8	7.39E-11
AW116075	HYDROXYSTEROID (17-BETA) DEHYDROGENASE 12A	-0.438	-1.3	4.27E-02
Losna ovary				
AF406784	VITELLOGENIN 1	0.930	1.9	0.0788
Mjøsa liver				
AF274877	ARYL HYDROCARBON RECEPTOR INTERACTING PROTEIN	-0.551	-1.5	0.0001
Mjøsa ovary				
AF406784	VITELLOGENIN 1	1.374	2.6	0.0014
AI545930	VITELLOGENIN 1, LIKE	1.349	2.5	0.0040
AF183906	CYTOCHROME P450, FAMILY 19, SUBFAMILY A, POLYPEPTIDE 1A	0.662	1.6	0.0469
BG308728	FK506 BINDING PROTEIN 5	0.438	1.4	0.0178
U55863	ZONA PELLUCIDA GLYCOPROTEIN 3 B	-0.404	-1.3	0.0483

Table 12: *Of the 660 significantly different regulated mRNA transcripts (Table 10), 20 were selected that are of special interest because they are involved in processes known to be affected by components of these mixtures, after exposure to mixtures harvested from Lake Mjøsa and Lake Losna. Genes in red typing are up-regulated while genes in green typing are down-regulated. GeneBank accession numbers are given together with the gene names and the P-values are adjusted so that the results do not exceed a false discovery rate of 10 %.*

5. Discussion

The results suggested that exposure of zebrafish to environmental relevant concentrations of real life mixtures of POPs affected demographic variables as well as specific gene transcripts and proteins.

5.1 Bioaccumulation and biomagnification of the mixtures

5.1.1 Correlation between POPs in Artemia nauplii and zebrafish

Exposure to the Mjøsa and Losna mixtures resulted in accumulation of the same toxins in artemia and zebrafish as in burbot liver (Table 3 and 5-8), and BDE-47 was the most abundant congener in artemia fed to all the toxin exposed groups. There were, however, some exceptions. HCHs and Chlordanes were detected at low levels in Losna exposed artemia, though they were below the detection limit in artemia exposed to all three dose levels of the Mjøsa mixture. This difference was, however, not observed in the livers of burbots caught in Lake Mjøsa or Lake Losna, nor in the zebrafish exposed the Mjøsa and Losna mixtures, and is thus difficult to explain. Also, HBCD was not detected in Losna, Mjøsa low and Mjøsa medium exposed artemia, though accumulated levels were observed in burbot livers and in the zebrafish treated with both mixtures.

There was a good correlation between the toxins that were found accumulated in livers of burbots from the two lakes and those observed accumulated in the artemia and zebrafish after exposure to the two mixtures (Fig. 14), and the difference in concentrations of BDE-47 in the artemia treated with the dose levels of Mjøsa mixture were not far from what they were exposed with, (medium 1/6 and low 1/22 compared to 1/8 and 1/32 of the high dose). (Muirhead et al. 2006) previously reported an uptake of a PBDE in Artemia nauplii, and they also observed an accumulation of BDE-47 in artemia after treatment for 24 hours and a biomagnification in Japanese Medaka (*Oryzias latipes*) and Fathead minnows (*Pimephales promelas*) following oral

exposure. The predictable transfer of toxins from artemia to fish observed in this and our study, suggest that artemia is an easy and reliable way of conduction exposure studies through dietary treatments.

5.1.2 Accumulation to environmental relevant levels of POPs

Looking at the concentrations of PBDEs measured in both artemia and zebrafish in this study, BDE-47 was the most abundant congener in all the exposed groups (Table 5 and 7), which was expected from both what we observed from the environment and from the pilot study, and is consistent with several other studies on wild freshwater and marine fish (Hartmann et al. 2007) and (Brown et al. 2006).

The mean BDE-47 levels measured in the different mixture treated groups (from 6 599 ng/g l/w in the Mjøsa High group to 93,6 ng/g in the Losna group) are also consistent with what has been reported earlier in fish at the same location (NIVA-4809 2004), as well as in other parts of the world; 2 800 ng/g to 17 000 ng/g in Maine (Anderson and Macrae 2006), n.d. to 4 370 ng/g in Swiss rivers (Hartmann et al. 2007), 19 ng/g to 565 ng/g in Spain (Labandeira et al. 2007), 6 ng/g to 160 ng/g in Canada (Ikonomou et al. 2002) and 164 ng/g in California (Brown et al. 2006). We also obtained levels of ΣPCBs and ΣDDTs ranging from 131 – 1 332 ng/g and 45,8 – 624 ng/g lipid weight respectively, which are similar to what observed in seal tissue from the northern gulf of Alaska (16 – 728 ng/g and 78 – 325 ng/g lipid weight for sum PCBs and DDTs respectively) (Wang et al. 2007). In addition, (Schiedek et al. 2006) reported of levels of PCBs ranging from 300 – 2 700 ng/g and a mean level of DDTs of 500 ng/g lipid weight in eels from the south western Baltic Sea.

The concentrations of BDE-47 in fish exposed to the Losna mixture and medium dose of the Mjøsa mixture are in the range of what have been reported in some members of the US population with reported levels of 2,9 to 272 ng/g in mother's milk (Schecter et al. 2003), 2,5 to 250 ng/g in blood plasma (Bradman et al. 2007) and 245 ng/g in serum (Fischer et al. 2006). Of the OCs, we measured levels of ΣPCB7 and DDE ranging from 68,5 – 774 ng/g and 27,1 – 551 ng/g lipid weight respectively (Table 8)

which are within the range of what reported in breast milk in Russian women, 69 – 680 ng/g for PCBs and 160 – 1 700 ng/g for DDE (lipid weights) (Tsydenova et al. 2007) and in Polish women, 35,0 – 485,9 ng/g and 493 – 2 519 ng/g (lipid weights) respectively (Szyrwinska and Lulek 2007). (Karrman et al. 2006) reported of similar levels in Swedish blood samples, ranging from 141 – 1 193 ng/g for PCBs and 29 – 895 ng/g for DDE (lipid weight). Thus, the concentrations of PBDEs and OCs obtained in our study can be related not only to levels observed in the wildlife, but also to what found in some members of the population.

Although we obtained environmental relevant concentrations in the fish, we were aiming for the Mjøsa Medium group to be at the same level as in livers of burbots caught in Lake Mjøsa, and the Mjøsa High group to be even higher than that. Instead, none of the Mjøsa treated fish reached levels as high as in the burbots and we suspect that the low hatching rate of the artemia was to blame, since much of the toxins must have been retained here.

It was unexpected that control and EE2 fish contained levels of OCs almost as high as the Losna treated fish, since the artemia they were given had very low levels. Further samples of artemia are intended to be analyzed to see whether it is possible to reproduce the results.

5.1.3 Biotransformation of BDE-99 in Zebrafish

The accumulation of BDE-99 observed both in liver oils of burbots from Lake Mjøsa and Lake Losna and in artemia exposed to the two mixtures, was not observed in any of the mixture treated zebrafish groups (Fig. 14). To our knowledge, this has not been reported earlier in zebrafish although it has been observed in wild common carp (Labandeira et al. 2007) and (Hale et al. 2001), which is also intriguing since BDE-99 is the dominant congener in the commercial PentaBDE mixtures. Thus, it seems that the non-existent accumulation observed in these species compared to burbots which accumulate BDE-99 are a result of efficient metabolism, maybe partly caused by

debromination of BDE-99 to BDE-47 as previously reported in carp (Stapleton et al. 2004), however, it remains to be investigated if this is also the case in the zebrafish.

5.2 Effects on demographic variables due to exposrue

5.2.1 A decrease in overall survival rate

In both generations, the survival was significant better in the Control group than in the groups exposed to the real life mixtures of POPs (Fig. 15 and 17). The indication is that the mixtures, independent of dose and origin, affected the survival of the fish. For the F_0 generation the effects observed must have been directly linked to the exposure that took place, while they for the F_1 generation may have been both directly and indirectly linked. Between 0-6dpf, the significant differences in survival observed between the F_1 eggs and larvae from mixture treated parents and from control parents must have been an indirect result of exposure since the F_1 larvae had not yet been exposed. Thus, the parents must have passed on something to their offspring that affected the survival ability in a negative way, indicating transgenerational effects. After day 6, the differences in survival could have been both directly and indirectly linked to the exposure and it seems that both were the case. The Control-Mjøsa high group showed a significantly higher mortality rate than the Mjøsa High-Mjøsa High group, and the same was true for the Control-Losna and Losna-Losna groups although the difference between these groups was not significant. These data suggested that the fish, if they survived the first six days, handled the exposure in a better way (i.e. better survival) if their parents had been subjected to the same treatment. This is in contrast to what (Brennan et al. 2006) reported in *Daphnia magna*, where crustaceans exposed to different oestrogens showed a weakening of the offspring in the first generation leading to increased mortality in the second generation at concentrations ranging from 0,2 to 1,0 mg/L. Also, (Mccoy et al. 1995) reported of lower survival in the second generation of mice exposed to low doses of Aroclor 1254, a commercial mixture of PCBs.

If our findings are biologically correct, there might be several reasons for the effects on the F_1 survival observed. Natural selection may have lead to survival of the strongest F_0 fish in the mixture exposed groups, which gave rise to strong and healthy offspring with increased survival ability. Another possibility might be that epigenetics are involved in the survival response to toxins. DNA methylations and histone modifications through methylations, demethylations, acetylations, deacetylations, phospholyations and ubiquitinations are involved in the activation and silencing of many genes without altering the primary DNA sequence. Epigenetic changes can arise within a generation and could therefore be responsible for the increased survival of the Mjøsa-Mjøsa and Losna-Losna groups compared to Control-Mjøsa and Control-Losna. Also, epigenetic changes can be inherited by offspring (Anway et al. 2005), and so it is possible that the parents (F_0) acquired these modifications during five months of exposure and passed them on to their descendants.

5.2.2 Males vs. Females

The overweight of males seen in the F_0 Control group (54,2 % males) is common in zebrafish populations (Brion et al. 2004) and reported in other exposure studies; 58,7 % males (Lin and Janz 2006) and 52,5 % males (Uchida et al. 2002).

The significant lower proportion of females seen in the Losna exposed group compared to the Control group (39,6 % vs. 45,8 %) and in the Mjøsa exposed groups though not significant, was not consistent with our other findings of an increase in the vitellogenin 1 mRNA levels in both Mjøsa and Losna mixture treated groups. However, the sex ratio in zebrafish seems to be unstable and sex determination has therefore been suggested to be polyfactorial (Brion et al. 2004).

5.2.3 Small differences in effects of the Mjøsa and Losna mixtures

We expected to see larger differences in the effects of the two mixtures than what we did during this study. First, they are two different mixtures with different compositions of toxins. The Mjøsa mixture contains high levels of PBDEs, while these compounds

were present at low levels in the Losna mixture. Second, Lake Losna is regarded as a much less polluted lake than Lake Mjøsa and the levels of the toxins measured in the Losna mixture were generally much lower than what observed in the Mjøsa mixture (Table 3). The effects of the Losna mixture were thus believed to be more similar to those of the control fish rather than those observed in the Mjøsa treated fish. It was therefore surprising to see that this was not the case. No significant difference was observed between the two mixtures in the survival of the F_0 generation (Fig. 15) and F_1 generation up to day 6 (Fig. 16). The Mjøsa high and Losna F_0 survival curve followed each other throughout the experiment and were at no time point significantly different. In F_1, the offspring from Losna exposed parents also showed the same survival rate as those from Mjøsa treated parents. After day 6 and onwards, however, the survival rate of the Control-Losna group was similar to that of the Control-Control group, although the Losna-Control had a significantly higher mortality rate. In addition, no significant difference in body mass was observed between the two mixtures (Fig. 19) and only Mjøsa High of the three Mjøsa exposed groups was significantly different from the Losna group in length (Table. 9). Also, the Mjøsa High and Mjøsa Medium groups showed no significant difference in EROD activity and Vtg induction compared to the Losna group (Fig. 14 and 15).

The reason for the apparent lack of agreement between the level of exposure and the observed effects may have several explanations and only speculations can be made. Higher levels of OCs were found in zebrafish treated with the Losna mixture than in those treated with the Mjøsa low mixture (Table 8), though the concentrations were still low and hardly the sole reason for the effects seen. Other than this, no distinct differences in the toxin compositions measured were found that could explain the effects, though one possibility could be that there are other contaminants involved in these effects which are not included in the chemical analysis in the present study. Another explanation is possible combinatorial effects of several of the components of the Losna mixture which may have acted synergistically when administered at such low doses, and thus having similar effects as the mixture of pollutants in the Mjøsa mixture. In addition, there may be other explanations to what we have observed besides those suggested here.

5.2.4 The many effects of EE2

The EE2 group was included in the experiment as a positive control for oestrogen exposure, and was affected in several different ways compared to the other groups. For instance, all the groups showed the same survival curve pattern, except the oestrogen treated group. A steep decline after 10dpf was observed also in this group, but fewer individual deceased and the steep decline flattened out earlier, at around day 19. However, in contrast to the other exposed groups, the oestrogen exposed fish showed a gradual decline in survival throughout the exposure period, with an increased rate towards the end (Fig. 15). It thus seemed that the oestrogen exposure had a protective effect on survival in the initial phase of the exposure period, but only then. In addition, the oestrogen treated fish were significantly smaller and shorter compared to the control fish (Fig. 19, Table 9) which was consistent with what (Lin and Janz 2006) reported. The total feminization observed in the oestrogen group exposed to 10 ng/L of EE2 was also reported by (Orn et al. 2006) while (Santos et al. 2006) observed the same with a concentration of only 3,5 ng/L. (Lin and Janz 2006) reported of males in the population when fish were exposed to 1 ng EE2/L or less. In addition, a Vtg concentration of 588 600 ng/ml was measured in the EE2 treated fish, which was almost 60 times as high as what measured in the control fish, and a 5 fold increase in induction compared to the highest concentration measured in any of the toxin treated groups. However, while Vtg induction was measured in males of the control and toxin treated groups, there were only phenotypic females present in the EE2 group and so Vtg levels had to be measured in females making it difficult to compare the results. Vtg concentrations in female control zebrafish have previously been reported to be as high as 300 000 ng/ml (Brion et al. 2004) suggesting that the high levels found in the EE2 group were a result of measuring in females. Also, (Van den Belt et al. 2003) reported of Vtg levels of 40 000 000 ng/ml in plasma of phenotypic female zebrafish exposed to 10 ng/L of EE2 for three weeks, indicating that higher levels than what observed could be expected. However, the Vtg levels measured in the oestrogen treated fish were at the borderline of the standard curve and may therefore not be as accurate as if the samples had been diluted further before conducting the assay.

In summary, long term exposure to the xenoestrogen EE2 at a concentration of 10 ng/L resulted in effects on survival, growth, sex ratio and vitellogenin induction different from what observed in the toxin exposed fish, and any possible oestrogenic effect of the mixtures are not nearly as strong as what observed in the oestrogen control group of this study.

5.3 Possible critical windows of exposure?

In this study, exposure occurred both when the larvae were developing and when they became adults, and it appears difficult to know if the toxin exposure affected the larvae more than adolescent of adult zebrafish. The drastic decline in survival that occurred between 10 and 22dpf was observed in all groups in both generations and was as such, unrelated to treatment. However, during the same time window exposure related effects were expressed by lower survival. After this period the survival rate stabilised in all groups irrespective of exposure. This indicates that exposure to toxins during that time period was the main reason for the differences observed in overall survival, and it might be a critical period for the larvae. The larvae start to eat at day 5 or 6, which may, at least partly, explain a critical period, and if they are not able to take in food because it is too large or the larva has a deformity in the mouth area they will eventually die. However, (Burkhardt-Holm et al. 1999) did not observe such a decline in survival in the control group nor the exposed groups (exposed to different doses of 4-chloroaniline from 0 to 16dpf) indicating that it might be specific to our zebrafish population rather than it being a critical period. The effects seen on the sex ratio may also be due to exposure at a critical period during juvenile development. Zebrafish are hermaphrodites with both sexes having undifferentiated ovary-like gonads the first three weeks of life. Sex differentiation starts between day 21 and 25 and females continue to develop the gonads into ovaries while males degenerate them and develops testicular tissue instead (Lin and Janz 2006) and (Uchida et al. 2002). (Brion et al. 2004) reported of an increase in phenotypic females after exposure to E2 from fertilization to day 21 and from day 21 to 42, indicating that oestrogen exposure may exert effects at both stages of development.

Thus, our study has revealed possible critical windows of exposure for survival and sex ratio in zebrafish, though further investigations must be conducted before any conclusions can be made. Also, in contrary to our experiments, most studies investigate possible critical windows of exposure during embryo development, and so few comparisons are available.

5.4 The EROD marker is less sensitive to mixture exposure

Both the Mjøsa and Losna mixtures led to significantly higher EROD activity compared to the controls, which was not unexpected considering that they both contain dioxin-like compounds reported capable of AhR mediated CYP1A induction (Safe 1993). More surprisingly though, it was exposure to the Mjøsa low dose that resulted in the highest EROD activity (0,0686 pmol resorufin/gill arch/min) compared to the Mjøsa medium and Mjøsa high dose (0,0609 and 0,0610 respectively, Fig. 20).

Although the differences in EROD activity between control and mixture treated groups were significant, they were not very large (between 1,13 and 1,22 fold increase). A study using the dioxin-like PCB-126, a known CYP1A inducer, reported a much larger increase in EROD activity of 74 fold after just 24 hours of exposure (Jonsson et al. 2006). Thus, it appears that neither of the two mixtures were strong CYP1A inducers when administered orally in environmental relevant concentrations. Although the highest measured PCB congener, PCB-153 and PCB-138, are non-planar, other known inducers are present in the mixtures, and so the results may be due to lower induction potency of the mixtures or catalytic activity of the protein. The lack of up-regulation of CYP1A mRNA in the liver gene expression analysis indicated that the mixtures had lower induction potential. Also, (Kuiper et al. 2004) reported that several of the BDE congeners had an antagonistic effect on dioxin induced EROD activity, possibly via the Ah receptor. This was later supported by (Peters et al. 2006) who showed that some PBDEs bound to the AhR but did not induce EROD activity because they were unable to activate the AhR-Arnt-XRE complex. The down-regulation of the AhR interaction protein gene observed in this study in liver of Mjøsa

treated fish, which associates with the ligand unbound AhR-Hsp 90 complex when translated, may indicate low levels of unbound AhR supporting those findings. Another possible explanation for the small differences in EROD activity could be that the assay was not sensitive enough or that the induction in the gills was much lower than in the liver after oral exposure. All publications on EROD measurements in gills have been conducted after waterborne exposure, not dietary, and (Pacheco and Santos 2001) reported no significant induction in gills after injection of 4 000 ng/g β-naphthoflevone (BNF), a potent inducer. However, if our observations were the result of assay insensitivity or low induction in the gills, we would expect to see CYP1A mRNA up-regulated in the livers with the microarray analysis, which we did not.

Yet another possible explanation for the low EROD activity may be related to what appeared like an acquired resistance to the components of the mixtures. Several studies have reported of low EROD activity in wild fish living in PCB contaminated areas (Nacci et al. 1999), (Elskus et al. 1999), (Brammell et al. 2004) and (Yuan et al. 2006), and (Roy et al. 2001) concluded that the resistance observed in their study was most consistent with single-generation acclimation rather than a multigenerational genetic adaptation. Also, (Orn et al. 1998) observed a decrease in hepatic EROD activity from week 4 to week 13 after oral exposure to a high dose of PCB mixtures. Resistance developed over just one generation indicates that different epigenetic mechanisms like DNA methylation, histone modifications and RNA interference or degradation may be involved in the AhR-mediated toxin response. Also, post-translational regulation of transcription factors involved in the AhR pathway may also be involved in CYP1A regulation, thus making resistance possible without mutations in the genome.

Independent of the reason, our results suggests that solely measuring the EROD activity may not be such a good indication of exposure to dioxin-like compounds when they are present in combination with different PBDE congeners in real life mixtures and after long term exposure.

5.5 Apparent lack of vitellogenin induction

To our surprise, only the Mjøsa Low of the mixture exposed fish showed a significant increase in Vtg levels compared to the controls after long term exposure. Inductions in Vtg concentrations of 3,3 fold, 2,1 fold and 1,6 fold were seen in the Mjøsa High, Mjøsa Medium and Losna groups respectively, however they were not significant. We expected to se a significant induction in Vtg after exposure to both mixtures because of the increase in Vtg mRNA levels observed in the microarray analysis. The reasons for the apparent lack of induction after mixture exposure are not known, although on explanation might be that our control fish contained higher levels of Vtg than what reported in male controls earlier (9 985 ng/ml compared to 100 ng/ml and 3 000 ng/ml) (Brion et al. 2004) and (Van den Belt et al. 2003). It may also be a possibility that some of the compounds present in the Mjøsa mixture had antagonistic effects with regards to Vtg induction, which worked stronger at higher doses, and thus explaining why lower levels of Vtg was found in the Mjøsa High and Mjøsa Medium groups than in the Mjøsa Low group. On the other hand, a lack of Vtg induction in juvenile Atlantic salmon was reported after short term exposure to Aroclor 1254, a commercial PCB mixture, *o'p'*-DDT (Arukwe et al. 2000), and the commercial Penta- and OctaBDE mixtures (Boon et al. 2002), supporting our findings. Thus it is difficult to evaluate whether our findings are biological or not.

5.6 Changes in overall gene expression patterns

5.6.1 Mixture specific differences

The Losna mixture regulated the levels of more mRNA transcripts than the Mjøsa mixture in the livers, while the opposite was true in the ovaries. Also, while the majority of the transcripts affected in the ovaries by the Losna mixture were also found regulated by the Mjøsa mixture, most of those regulated by the Mjøsa mixture were unique for that mixture. The opposite was true in the livers where the Mjøsa mixture regulated mostly mixture unspecific transcripts while the Losna mixture

regulated mostly mixture specific mRNAs (Fig. 14). These results indicate that the Mjøsa mixture (at least the high dose) had an elevated effect on the ovaries with regard to changes in gene expression pattern, while the Losna mixture had an elevated effect on the livers. The reason for and impact of this is difficult to understand, especially since no distinct differences in response were observed after treatment to the Losna and Mjøsa mixtures. Further processing of the microarray results might have given additional clues to the reason for this and possible effects.

Gene annotation and classification

The web accessible program DAVID was used to find the function of the regulated mRNA transcripts, however, there was several gene names DAVID did not recognize or was not able to assign a function (Table 9). The GeneBank accession numbers that were pasted into DAVID originated from the Array List that was used to identify each spot on the array. The accession numbers that were not recognized, because they were not present in the DAVID database, or not assigned a function might have been recognized or annotated by other annotation programs available which use other databases. Also, BLAST searches conducted with the unknown gene sequences towards other species might have resulted in more gene names being annotated.

Classification of the differently expressed mRNA transcripts was conducted to get a better understanding of the pathways affected by mixture treatment, however, the broad and overlapping categories did not give a clearer picture of the processes involved (Table 10). Assigning function to more of the differently expressed gene names might have identified more transcripts involved in effected pathways and thus given a clearer picture.

5.6.2 Effects from low changes in mRNA levels

None of the mRNA transcripts affected by exposure showed a larger change than 3 fold up- or down-regulation. It cannot be ruled out that technical variation might have been partly responsible but it may also be a biological result. We were not expecting any large toxic responses since the exposure was conducted with low, environmentally

relevant concentrations of the mixtures. It therefore seems that the effects observed are the result of only small changes in mRNA levels.

5.7 Specific genes of interest regulated by the mixtures

Of the genes selected because of interesting relations to known toxic responses, some were found regulated after exposure to the Losna mixture, some after exposure to the Mjøsa mixture and some were found regulated in both Mjøsa and Losna exposed fish.

5.7.1 Altered expression of a phase II biotransformation gene

Glutathione-S-transferase (GST) theta 1 mRNA was found down-regulated 1,5 fold in livers of Losna treated fish. As reviewed in (Frova 2006), glutathione conjugations are important detoxification steps which metabolise highly reactive compounds to make them more polar and water soluble. GST enzymes catalyze the conjugation of GSH with endogenous and exogenous compounds that contain a highly reactive electrophilic centre (Frova 2006). PCB exposure has previously been shown to increase hepatic GST activity (Machala et al. 1998) and (Schmidt et al. 2005) or have no significant effect (Mayon et al. 2006). However, (Bernhoft et al. 1994) reported in 1994 a decrease in hepatic GST activity following exposure to PCB-105 which is present in the mixtures although at low levels. The reported effects on GST activity described above are in none of the other studies observed through a decrease or increase in mRNA levels but through changes in enzyme activity, and (Lo et al. 2004) reported of another GST family member, pi 1, which undergo post-translational modification in the form of phosphorylation to increase its metabolic activity.

Down-regulation of phase II biotransformation genes, and most likely their enzyme levels, can be dangerous because cells are no longer able to defend themselves in the same way against reactive toxic compounds which are harmful to the cells and may cause cancer or necroses.

5.7.2 Down-regulation of zona pellucida mRNA by the Mjøsa mixture

Treatment with the Mjøsa mixture resulted in down-regulation of zona pellucida 3B mRNA in ovaries. Zona pellucida are glycoproteins that make up the non-cellular membrane layer of fish and mammalian eggs, and are responsible for spermatozoa binding and may be required for initiation of the acrosome reaction where the spermatozoon releases its content into the egg. While other zona proteins, like zona radiata, have been shown to be induced by oestrogens (Berg et al. 2004), zona pellucida 3 mRNA in ovaries is not (van der Ven et al. 2006) and (Liu et al. 2006). To our knowledge, inhibition of zona pellucida mRNA in ovaries after exposure to different POPs has not been reported earlier. No knockdown studies have been performed on this gene, but total knockout of all the three zona pellucida genes in mice resulted in infertility (Naz and Rajesh 2005). Possible effects of our observation may be related to fertilization success, hatching ability and survival of the offspring, but also physiological changes in the ovaries regarding oocyte development and maturation.

5.7.3 Heavy metal response in the Losna exposed fish?

Metallothionein 2 mRNA was found up-regulated in livers of Losna treated fish. Metallothioneins (MTs) are cystein rich proteins that bind physiological and xenobiotic heavy metals such as sink (Ze), copper (Cu) and selenium (Se) and cadmium (Cd), mercury (Hg) and argon (Ar) respectively. MTs also control oxidative stress, which is caused by an increase in reactive oxygen species such as oxygen radicals and hydrogen peroxide (H_2O_2). The zebrafish metallothionein 2 gene is reported to contain four metal regulatory elements and three activator protein 1 (AP1) and one specific protein 1 binding sites in its promoter, and is induced by Zn^{2+}, Cd^{2+}, Cu^{2+} and Hg^{2+} ions with decreasing potential. In spite of containing three AP1 sites, which have previously been shown to mediate oxidative stress response (Samson et al. 2001), a change in expression after H_2O_2 treatment was not observed in that study (Yan and Chan 2004). This indicates that the zebrafish metallothionein 2 gene

responds to heavy metals more than oxidative stress. In contrary to our findings, (Gerpe et al. 1998) reported a reduction in metallothionein mRNA levels after exposure to three PCB congeners (OH-PCB-30, PCB-104 and PCB-190), however these congeners have not been measured and it is thus unclear if they are present in the mixtures. Chemical analysis for the presence of heavy metals is currently being conducted by the Veterinary Institute.

5.8 Oestrogenic effects of the real life mixtures

The 2,3, 1,9 and 2,6 fold increase in Vtg mRNA observed in livers and ovaries of Losna treated fish and ovaries of Mjøsa treated fish respectively, indicate that both the Losna and Mjøsa mixtures possessed oestrogenic effects. To our surprise however, this was not fully supported by the Vtg biomarker assay where only Mjøsa low treated fish showed a significantly different induction compared to the controls. The Vtg precursor proteins are post-translationally processed in the oocytes (Nicolas 1999) which could possible be a target for regulation, however, our findings of Vtg induction at the mRNA, and to some extent at the precursor protein level, supports that regulation at least occur at the gene level.

Up-regulation of CYP19A1 mRNA found in ovaries of Mjøsa treated fish, codes for the enzyme aromatase that convert androgens to oestrogens and contributes to an increase in E and E2 levels. CYP19A1 is the predominant CYP19 in zebrafish gonads and its promoter is reported to contain the AhR recognition site, cAMP-responsive elements and a steroidogenic factor I site (Kazeto et al. 2001). Toxin exposure leading to ligand bound AhR-Arnt complex may therefore contribute to increased CYP19A1 transcription, although in mice, the complex must cooperate with an orphan nuclear receptor Ad4BP/SF-1 to be able to activate transcription in ovarian granulosa cells (Baba et al. 2005).

In livers of Losna treated fish, the down-regulation of cytosolic sulfotransferase (SULT) 2 and 6 mRNA may contribute to increased oestrogen activity. As reviewed in (Coughtrie et al. 1998), sulphate conjugations detoxify exogenous compounds,

however, they are also responsible for the sulfonation of endogenous steroid hormones, leading to their inactivation (Strott 2002). The enzymes catalyze the transfer of sulfuryl groups from PAPS to the various substrates. The two sulfotransferase (SULT) isoforms 2 and 6 are members of the phenol sulfotransferase (SULT1) family of SULTs, though they are both shown to be involved in sulphating of the endogenous oestrogens E and E2 (Ohkimoto et al. 2004) and (Yasuda et al. 2005). In addition, the SULT isoform 2 is also capable of sulphate conjugating the thyroid hormones T_4 and T_3 and dehydroepiandrosterone (Ohkimoto et al. 2004). Two studies published in 2006 and 2007 reported that some OH-PCB were able to inhibit SULT1A1 activity, the human ortholog to SULT isoform 2, (Wang et al. 2006) and E2 sulfonation (Wang and James 2007), but the mixtures have not been analysed for the presence of OH-PCBs. The down-regulation of 17-β hydroxysteroid dehydrogenase 12A mRNA, a reductive enzyme which converts E into E2 (Luu-The et al. 2006), observed in livers of Losna treated fish may be a response to an increase in oestrogen activity.

As reviewed in (Bulun et al. 2005), over expression of aromatase and an increase in oestrogens is connected to human breast cancers, the most frequent cancer type in women all over the world. However, the hypothesis that the oestrogenic effects of OCs after natural exposure would cause breast cancer was reviewed in (Ahlborg et al. 1995) and not supported. Also, (Safe and Zacharewski 1997) concluded that the oestrogenic contribution from such compounds was too small compared to dietary intake of hormones to play a role in development of breast cancer. However, many methods for evaluating oestrogen potency focus on compounds affinity for the oestrogen receptor as in enzyme-linked reporter assays (Seifert 2004) or on the proliferative effects of oestrogens as in E-screens (Soto et al. 1995), but there are several other mechanisms by which compounds can have oestrogenic effects, such as through production, transport and metabolism of endogenous oestrogens as reviewed in (Brouwer et al. 1999) Thus, a contribution of even low-dose exposure of xenoestrogens to the oestrogen-linked effects seen in the population, such as earlier fertility in females and decreasing male to female sex ratio at birth can not be ruled out.

5.9 Other endocrine disrupting effects of the mixtures

Several of the differently regulated mRNA transcripts in livers of Losna treated fish, and one in livers of Mjøsa treated fish, indicate that the mixtures have other effects on the endocrine system besides reproductive. For instance, CYP3A65 mRNA levels were found down-regulated in livers of Losna treated fish. CYP3A enzymes are responsible for the metabolism of many endogenous and xenobiotic compounds, and one of their important tasks is to terminate steroid hormone activity, such as that of testosterone (TST), androstenedione and progesterone, by converting the hormone into metabolites. CYP3A4, the human ortholog to zebrafish CYP3A65 (Tseng et al. 2005), is shown to metabolize TST into 6 β-hydroxytestosterone and 4-hydroxyandrostenedione (a potent inhibitor of aromatase) (Usmani et al. 2003). Mammalian CYP3A expression does not seem to be connected to the AhR, but respond rather to phenobarbital inducers, which are also known to be present in the mixtures, and so a CYP3A mRNA increase would be expected. However, (Tseng et al. 2005) reported a dioxin enhanced zebrafish CYP3A65 expression through the AHR/Arnt signalling pathway, indicating that xenobiotic induction of CYP3A65 is regulated in a different manner in zebrafish. The same study also showed a decrease in CYP3A65 mRNA after treatment with high dose of dexamethasone, a synthetic glucocorticoid, which is more consistent with what we observed. An increase in active steroid hormones was supported by the up-regulation of FK506 binding protein (FKBP) 5 and 11 mRNAs observed in livers of Losna treated fish and ovaries of Mjøsa treated fish (only FKBP5). The translated FKBP5 protein is a component of the steroid receptor complex and contributes to hormone desensitization by inhibiting the receptor activity. The gene contains two similar consensus sequences to the hormone response element in intron E, which bind and respond to both glucocorticoids and progesterone *in vitro* in humans (Hubler and Scammell 2004), and six androgen responsive elements downstream of the transcription initiation site, with two highly conserved in intron 5, which bind and respond to the androgen receptor *in vivo* in humans (Magee et al. 2006). Thus, transcription regulation of FKBP5 seems to occur through distal enhancers activated by steroid hormones. In contrary, little is known of

the FKBP11 gene other than that it, when translated, is a component of the progesterone receptor.

Steroid hormones are active only through their respective receptors and are involved in their regulation through positive or negative feedback loops. As reviewed in (Ing 2005), this is also done by a stabilisation or destabilisation of the receptors mRNA transcripts, and is most likely the reason why none of these receptor genes were found up- or down-regulated in the microarray analysis.

Deiodinase, iodothyronine type II mRNA was also found down-regulated in livers of Losna treated fish, and this enzyme is responsible for converting the thyroid hormone T_4 into T_3, which is the more bioactive thyroid hormone (three to four times more potent than T_4). A study conducted by (Shirey et al. 2006) on tadpoles reported a decrease in deiodinase type II mRNA after exposure to the commercial PCB mixture Aroclor 1254, while two studies have shown a decrease in T3 levels in seals exposed to PCBs and DDTs (Sormo et al. 2005) and (Debier et al. 2005) supporting our findings. Also, a decrease in LDL receptors, which leads to cholesterol depletion in the cells, is associated with T_3 depletion (Ness et al. 1990) which was supported by the up-regulation of sterol-C4-methyl-oxidase-like (SMO) mRNA found in livers of Losna treated fish, which when translated is an enzyme involved in sterol biosynthesis. The gene is regulated upon cholesterol depletion by the transcription factor sterol regulatory element binding protein (SREBP), which translocates to the nucleus after being cleaved by proteases and binds to conserved promoter motifs called sterol regulatory elements (Wang et al. 1994), and regulate genes involved in cholesterol uptake and synthesis. Such a SRE has been identified in the promoter of the SMO yeast homolog ERG25 (Vik and Rine 2001). Contrary to what may be expected, SRBP mRNA was not among the up-regulated genes, however, this protein can be regulated at a post-translational level by an increase in activity of the proteases that cleaves it free from the endoplasmatic reticulum membrane (Wang et al. 1994) thus explaining our observations. Consistent with our findings, (Shin and Osborne 2003) reported that an increase in SREBP nuclear protein was directly linked to low levels of T_3 thus identifying the hormone's indirect regulation mechanism of the LDL

receptor. A decrease in LDL receptors causes an increase in plasma cholesterol levels, which is associated with hypercholesterolemia. Hypercholesterolemia may lead to atherosclerosis and increased risk of cardiovascular diseases (Soutar and Naoumova 2007) which are the primary cause of death and disability in the US and most European countries today.

Angiotensinogen mRNA was also found up-regulated in livers of Losna treated fish. The translated angiotensinogen protein (AGT) is a precursor to angiotensin I, which, after conversion to angiotensin II by angiotensin-converting enzyme (ACE), causes the release of aldosterone from the adrenal cortex and a subsequent change in the salt balance. AGT transcription is reported to be regulated by several factors such as glucocorticoids, oestrogens, androgens, thyroid hormones, angiotensin II and cytokines both *in vitro* and *in vivo* (Stavreus-Evers et al. 2001) and (Lynch and Peach 1991). An up-regulation of ACE mRNA was not observed which indicate that there is no increase in angiotensin II protein, unless the enzyme can be regulated at other levels. Keeping elevated concentrations of AGT mRNA will make the response time much shorter when angiotensin II is needed.

The difference in mRNA levels observed in this study supports our expectations that the mixtures would have endocrine disrupting effects. As indicated above and reviewed in (Longnecker et al. 2003), there is a growing realization that low-level exposure to xenobiotics can cause endocrine disrupting effects in humans, partly because of observed physical changes in the population connected to the endocrine system and partly because of increased knowledge of the toxic potential of compounds.

6. Concluding remarks

The main aim of the study was to look at the effects of long-term exposure to environmental relevant concentrations of real life mixtures of POPs in the zebrafish. We have found direct physiological effects relating to the survival and growth of the fish after exposure to both the Mjøsa and Losna mixtures, indicating harmful effects of them both. Effects observed were only partly consistent with what reported from single chemical exposure supporting the suggested difficulty in predicting mixtures potencies. The traditional biomarker assays indicating dioxin-like and oestrogen exposure were only partly induced, and no large differences were observed between the Mjøsa and Losna mixtures. The zebrafish has been shown to be a good model for this kind of toxicogenomics studies, and the indirect exposure through the diet proved both predicable and easy to conduct.

Microarray analysis revealed some mixture dependent alterations in gene expression patterns, although elevated levels of vitellogenin mRNA were found after exposure to both the Mjøsa and Losna mixture. Elevated levels of vitellogenin mRNA and some other mRNA transcripts indicated an oestrogenic effect of both the mixtures. Also, several mRNA transcripts affecting steroid hormones, directly or indirectly, were found differently regulated indicating other endocrine disrupting effects as well.

Results indicated possible critical windows of exposure related to survival and sex ratio of the fish. Exposure during a drop in survival between day 10 and 20 seemed to be the major factor for the differences in overall survival observed, while exposure during the start of sexual development around day 20 may have played a role in the final sex distribution. The effects on F_1 survival observed before and after exposure also indicated transgenerational effects of the two mixtures.

In summary, environmental relevant concentrations of the real life mixtures harvested from burbots in Lake Mjøsa and Lake Losna both showed direct effects on survival and growth of the fish, as well as effects linked to development and reproduction.

7. Future studies

The Mjøsa Low group differed in both EROD activity and Vtg induction compared to the other mixture treated groups and if microarray analyses are performed on these samples results might reveal some answers to this. Also, gene expression analysis of the EE2 exposed fish might reveal information about the oestrogenic effects on mRNA levels which could be compared to the gene expression patterns already obtained. Further investigations of the possible critical windows of exposure should be performed on later generations, especially with regards to the steep decline in survival, which has not been reported earlier, and the lack of BDE-99 accumulation observed in the zebrafish should be studied in more detail. In addition, further investigation of the regulated mRNA transcripts by using other annotation tools and BLAST searches may identify more of the genes and give a more complementary result.

Verification of microarray data using other methods is considered a requirement by today's standards and should be performed on at least some of the results obtained, and qRT-PCR which measures specific mRNA levels in samples is used to verify data on the mRNA level. Also, since regulation mechanisms in cells occur not only at the transcriptional level but also at the translational and protein level, verification through protein detection by western blotting should also be performed.

The identification of new biomarkers, which may be used in the detection of exposure to low doses of mixtures of toxins possessing a health risk, would be valuable and time saving, and transgenic indicator organisms could be produces telling us when such a pollution is present. Thus, further investigation of the mixture specific and unspecific mRNA transcripts regulated in this study might reveal some candidate genes.

At some point the real life mixtures must be recreated synthetically so that there is no possibility of unknown factors affecting the results. This is the easiest way one can say with certainty that the components measured in the mixtures are the ones responsible for the observed effects.

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8. Appendix

8.1 Solutions

8.1.1 EROD assay

HEPES-Cortland buffer:

KCl	0,380 g
NaCl	7,740 g
$MgSO_4$ x $7H_2O$	0,230 g
CaCl x $2H_2O$	0,230 g
NaH_2PO x $2H_2O$	0,372 g
HEPES	1,430 g
Glucose	1,000 g

Distilled water up to 1 L and pH adjusted to 7,7 with 1 M NaOH

EROD buffer:

HEPES-cortland buffer, pH 7,7	35 ml
Dicoumarol (1 mM)	35 µl
7-ethoxyresorufin (10 mM)	70 µl

8.1.2 Vtg assay

Homogenization buffer:

Tris-HCl, pH 7,4	12 ml
Protease inhibitor cocktail	120 µl

8.1.3 Microarray analysis

Pre-hybridization buffer:

0,1 % Bovine Serum Albumin (BSA) Fraction B

5 x SSC (25 ml 20 x SSC: 3 M NaCl, 0,3 M sodium citrate, pH 7,0)

0,1 % SDS (1 ml of 10 % SDS

100 mg of BSA fraction V are dissolved in 70 ml of double distilled water under gentle stirring. SSC and SDS are added together with double distilled water to 100 ml.

Wash solution 1:

0,5 x SSC (25 ml 20 x SSC)

0,01 % SDS (1 ml 10 % SDS)

dH_2O added to 1 L

Wash solution 2:

0.06 x SSC (3 ml 20 x SSC)

dH_2O added to 1 L