Exposure of Zebrafish to Brominated Environmental Chemicals

Studies on reproduction, maternal transfer and early life-stage development

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Abstract

Fish reproduction and embryonic development may be sensitive to environmental chemicals. This thesis investigated effects of brominated flame retardants (BFRs) and polybrominated dibenzo-*p*-dioxins (PBDDs) on reproduction and early life-stage development in zebrafish (*Danio rerio*). Most of these compounds are highly hydrophobic, hence it was hypothesized that important routes of exposure are dietary uptake and maternal transfer.

Adult zebrafish were exposed to feed spiked with a mixture of structurally diverse BFRs to investigate accumulation from feed, maternal transfer, and effects on reproduction and early life-stage development. One of the compounds in the BFR mixture, i.e. 2,4,6-tribromophenol, was tested separately. The BFRs were also tested individually in an embryo toxicity test, to screen for effects of waterborne BFRs on early life stages. To investigate effects of PBDDs on reproduction, early life-stage development, and on the aryl hydrocarbon receptor (AHR) pathway, adult zebrafish were exposed to feed spiked with 2,3,7,8-tetraBDD (TBDD), or a mixture of PBDDs that was designed to reflect relative concentrations found in Baltic Sea biota.

Most brominated chemicals exposed via feed were detected in females and in their offspring. Ovarian morphology was altered in all studies, and the PBDDs induced AHR-regulated genes and ethoxyresorufin-*O*-deethylase activity. Effects on early life-stage development were seen after parental and water exposure, although at concentrations generally higher than in the environment. However, compared to several other fish species, zebrafish have a relatively low sensitivity to AHR agonists regarding effects on early life stages. To be able to evaluate the risk of PBDDs for fish in the Baltic Sea, future studies should focus on fish species native in the Baltic Sea. Overall, the results suggest that maternal transfer is an important exposure route for several brominated environmental chemicals, and that these compounds may interfere with reproduction and early life-stage development in fish.

Keywords: zebrafish, reproduction, early life-stage development, maternal transfer, ovarian histopathology, brominated flame retardants, brominated dioxins, toxicity

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Dedication

Till hela min familj, men extra mycket till Lisa och Sara

Contents

List of Publications

This thesis is based on the work contained in the following paper, referred to by Roman numerals in the text:

I Jenny Rattfelt Nyholm, Anna Norman, Leif Norrgren, Peter Haglund, Patrik L. Andersson (2008). Maternal transfer of brominated flame retardants in zebrafish (*Danio rerio*). *Chemosphere* 73(2), 203-208.

II Anna Norman Haldén, Jenny Rattfelt Nyholm, Patrik L. Andersson, Leif Norrgren. Reproductive and developmental effects in zebrafish (*Danio rerio*) exposed to structurally diverse brominated flame retardants (manuscript).

III Anna Norman Haldén, Jenny Rattfelt Nyholm, Patrik L. Andersson, Henrik Holbech, Leif Norrgren (2010). Oral exposure of adult zebrafish (*Danio rerio*) to 2,4,6-tribromophenol affects reproduction. *Aquatic Toxicology* 100(1), 30-37.

IV Anna Norman Haldén, Kristina Arnoldsson, Peter Haglund, Anna Mattsson, Erik Ullerås, Joachim Sturve, Leif Norrgren. Retention and maternal transfer of brominated dioxins in zebrafish (*Danio rerio*) and effects on reproduction, aryl hydrocarbon receptor-regulated genes, and ethoxyresorufin-*O*-deethylase (EROD) activity (manuscript submitted).

Abbreviations

1237-TeBDD	1,2,3,7-tetrabromodibenzo-*p*-dioxin
1238-TeBDD	1,2,3,8-tetrabromodibenzo-*p*-dioxin
1247/1248-TeBDD	1,2,4,7/1,2,4,8-tetrabromodibenzo-*p*-dioxin
1368-TeBDD	1,3,6,8-tetrabromodibenzo-*p*-dioxin
1379-TeBDD	1,3,7,9-tetrabromodibenzo-*p*-dioxin
137-TrBDD	1,3,7-tribromodibenzo-*p*-dioxin
138-TrBDD	1,3,8-tribromodibenzo-*p*-dioxin
1-MBDD	1-monobromodibenzo-*p*-dioxin
237-TrBDD	2,3,7-tribromodibenzo-*p*-dioxin
27/28-DBDD	2,7/2,8-dibromodibenzo-*p*-dioxin
AHR	aryl hydrocarbon receptor
ANOVA	analysis of variance
BDE 183	2,2',3,4,4',5',6-heptabromodiphenyl ether
BDE 209	decabromodiphenyl ether
BDE 28	2,4,4'-tribromodiphenyl ether
BDE 47	2,2,4,4-tetrabromodiphenyl ether
BrSty	2-bromostyrene
CYP1A	cytochrome P4501A
decaBDE	technical product containing mainly BDE 209
EDCs	endocrine disrupting chemicals
ELISA	enzyme-linked immunosorbent assay
EROD	ethoxyresorufin-*O*-deethylase
FSTRA	Fish Short Term Reproduction Assay
GI	gastrointestinal tract
GR	glutathione reductase
GtH	gonadotropins
HBCD	hexabromocyclododecane
hpf	hours post fertilization
HPVC	high production volume chemical
HxBrBz	hexabromobenzene
K_{ow}	octanol-water partition coefficient
LOEC	lowest observed effect concentration
NOEC	no observed effect observation
octaBDE-mix	technical product containing hexa- to decabrominated diphenyl ethers
OECD	Organization for economic co-operation and development
PBDDs	polybrominated dibenzo-*p*-dioxins

PBDEs	polybrominated diphenyl ethers
PCBs	polychlorinated biphenys
PCDDs	polychlorinated dibenzo-*p*-dioxins
pentaBDE-mix	technical product containing tri- to hexabrominated diphenyl ethers
real-time RT PCR	real-time reverse transcription polymerase chain reaction
TBA	2,4,6-tribromoanisole
TBBPA DBPE	tetrabromobisphenol A 2,3-dibromopropyl ether
TBBPA OHEE	tetrabromobisphenol A 2-hydroxyethyl ether
TBBPA	tetrabromobisphenol A
TBDD	2,3,7,8-tetrabromodibenzo-*p*-dioxin
TBECH	1,2,-dibromo-4-(1,2-dibromoethyl)cyclohexane
TBP	2,4,6-tribromophenol
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
Vtg	vitellogenin

1 Introduction

1.1 General background

Many chemicals released from human activities eventually reach the aquatic environment. Fish may be exposed to these anthropogenic chemicals via several routes, e.g. from water via the gills and the skin, and from food and ingested water via the gastrointestinal tract (GI). In the environment, hydrophobic (lipid soluble) chemicals tend to adsorb to particles and sediments, and they may bioaccumulate in animals, including organisms that serve as diet for fish. The hydrophobicity of a compound is measured by the octanol-water partition coefficient (K_{ow}). The importance of uptake of chemicals via the GI, relative to uptake via gills and skin, generally increases with increasing log K_{ow}, and compounds with log K_{ow}>6 are considered to mainly be taken up via the GI (Heath, 1995). Fish early life stages may be exposed to chemicals via maternal transfer, i.e. from the female tissues to the oocytes during oocyte maturation, or via uptake from water through the chorion. Maternal transfer has been reported for hydrophobic compounds (Ostrach *et al.*, 2008; Serrano *et al.*, 2008; Heiden *et al.*, 2005; Monteverdi & DiGiulio, 2000; Russell *et al.*, 1998; Ungerer & Thomas, 1996; Miller, 1993; Spies & Rice, 1988), whereas uptake from water via the chorion has been suggested to decrease with hydrophobicity (Braunbeck *et al.*, 2005).

Reproduction and early life stages are critical periods for exposure to environmental chemicals. A multitude of environmental chemicals have been shown to alter the reproduction in fish, such as dioxin-like compounds, which act via the aryl hydrocarbon receptor (AHR), and compounds that interfere with the endocrine system, i.e. endocrine disrupting chemicals (EDCs). In recent decades, several reproductive disorders in wild fish have been reported, such as reduced ovarian weight,

reduced ovarian maturity, intersex, skewed sex ratios, and early life-stage mortality. Exposure to chemicals that interfere with the reproductive system is one of several causes discussed. This thesis investigated effects of brominated environmental chemicals on reproduction in zebrafish (*Danio rerio*). The focus was on reproductive output, female gonad morphology and early life-stage development.

1.2 Regulation of fish reproduction

Fish reproduction is complex and includes a series of hormonally regulated biochemical responses. Moreover, environmental factors, such as temperature and photoperiod have been shown to regulate the reproductive cycle in fish (De Vlaming, 1972). The hormonal control of reproduction involves a hypothalamus-pituitary-gonadal-axis. In brief, the brain produces gonadotropin-releasing hormone, which stimulates the secretion of gonadotropins (GtH) in the pituitary. In fish, there are two GtHs: GtH-I and GtH-II (Swanson *et al.*, 1991; Kawauchi *et al.*, 1989). Gonadotropins are responsible for stimulating the production of sex steroids, i.e. estrogens and androgens, within the gonads of both sexes. The sex steroids, in turn, regulate different reproductive processes, e.g. gametogenesis, sexual phenotype, and reproductive behavior by complex feedback mechanisms of the hypothalamus-pituitary-gonadal-axis (Arcand-Hoy & Benson, 1998). Thyroid hormones, which are under the control of the hypothalamus and pituitary, and are involved in regulation of growth and development in fish (Power *et al.*, 2001), have been suggested to be involved in reproduction processes, such as oocyte and testicular growth (Cyr & Eales, 1996).

1.3 Ovarian development

The major developmental periods during oocyte growth are as follows: primary oocyte growth, cortical alveolus stage, vitellogenesis, and maturation (Tyler & Sumpter, 1996). The vitellogenesis accounts for the largest increase of oocyte growth and is controlled and regulated by hormones. During vitellogenesis in fish, large amounts of extra ovarian proteins are included into the oocytes and stored as yolk which serves as nutrition for the growing embryo and larval stages. The major yolk precursor, the lipoglycophosphoprotein vitellogenin (Vtg) (Tyler *et al.*, 1991), is produced in the liver. The production of Vtg is induced by ovarian estrogens, which in turn are stimulated by GtH-I. The Vtg is transported via the blood to the ovary, taken up into oocytes by receptor-mediated

endocytosis, cleaved into yolk proteins, and stored in yolk granules (Wallace & Selman, 1990; Wallace, 1985; Ng & Idler, 1983). Vtg uptake in oocytes is suggested to be stimulated by GtH-I (Tyler *et al.*, 1991), whereas maturation and ovulation of oocytes is proposed to be regulated primarily by GtH-II (Clelland & Peng, 2009; Swanson *et al.*, 1991; Scott & Sumpter, 1983).

The number of oocytes spawned, i.e. the fecundity, has in fish been suggested to be determined by genetics and nutritional status and, in the end, by the balance between number of mature oocytes and the degree of degeneration and resorption of oocytes (atresia) (Tyler & Sumpter, 1996). Follicular atresia involves hypertrophy of the granulosa and theca cells, folding and perforation of the chorion, and disorganization of the ooplasm, and may occur in follicles at any stage of oocyte development (OECD, 2009b; Nagahama, 1983). Atresia in fish is to some extent a natural process controlling fecundity, but atresia in the later stages of oocyte development, i.e. from the cortical alveolus stage, is considered to result mainly from environmental stress (Tyler & Sumpter, 1996).

1.4 Maternal transfer

During oocyte maturation, large amounts of circulating lipoproteins, e.g. Vtg, are transported from female tissues to the oocytes. Chemicals may bind or associate with these lipoproteins and thereby reach the oocyte (Monteverdi & Giulio, 2000; Ungerer & Thomas, 1996). Maternal transfer has been reported for various lipophilic organohalogen compounds, such as DDT, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Ostrach *et al.*, 2008; Serrano *et al.*, 2008; Heiden *et al.*, 2005; Monteverdi & DiGiulio, 2000; Russell *et al.*, 1998; Ungerer & Thomas, 1996; Miller, 1993; Spies & Rice, 1988).

1.5 Early life-stage development

An initial step in the fertilization process is activation of the egg, which involves a rapid increase in intracellular calcium concentrations. In mammals and amphibians, activation of the egg is triggered by the sperm contact. In fish, egg activation seems to be species specific. In zebrafish, activation occur when the egg comes in contact with the spawning medium rather than when the sperm enter the egg (Lee *et al.*, 1999). If activated, even unfertilized zebrafish eggs undergo early developmental steps but only fertilized eggs reach the four-cell stage (Lee *et al.*, 1999; Schulte & Nagel,

1994). The embryonic period in fish includes all stages from fertilization to the start of external feeding, i.e. the embryogenesis, hatching, and the yolk sac period (Balon, 1975). In zebrafish, the time from fertilization to hatching is about three days, and the yolk sac period ends around day 6 post fertilization (OECD, 2006; Kimmel *et al.*, 1995).

1.6 Effects on reproduction and early life-stage development

1.6.1 Reduced reproductive output

Reduced reproductive output, e.g. fecundity and fertilization, could result in population declines, and are thus ecologically relevant parameters. However, these parameters are difficult to monitor in wild fish. Under laboratory conditions, reduced fecundity, fertilization, and spawning success have been reported after exposures of adult fish to various EDCs, such as anti-estrogenic, estrogenic, androgenic, and aromatase inhibiting compounds (Van der Ven *et al.*, 2007; Ankley *et al.*, 2005; Tilton *et al.*, 2005; Pawlowski *et al.*, 2004; Ankley *et al.*, 2002; Van den Belt *et al.*, 2001). Reduced fecundity has also been observed after exposure of adult fish to organohalogens compounds, such as TCDD and 2,2,4,4-tetrabromodiphenyl ether (BDE 47) (King Heiden *et al.*, 2006; Muirhead *et al.*, 2005).

1.6.2 Altered ovarian development

There are several examples of altered ovarian development in wild fish in Sweden and in the Baltic Sea area. For example, decreased relative ovarian weight and maturity have been observed in perch (*Perca fluviatilis*) from Baltic Sea coastal areas affected by bleached kraft mill effluents (Andersson *et al.*, 1988; Sandström *et al.*, 1988), but also from coastal areas of the Baltic Sea with low local exposure to anthropogenic substances and low human population density (Hansson *et al.*, 2006). Moreover, a large frequency of non-spawning burbot (*Lota lota*) with immature ovaries has been described in the northern part of the Bothnian Bay (Pulliainen *et al.*, 1992). In the burbot, several environmental toxicants were detected, such as TCDD, but no clear cause-and-effect relation was established. Decreased relative ovarian weight, decreased maturity, and atresia have also been described in perch from a Swedish lake (Lake Molnbyggen) close to a public refuse dump (Linderoth *et al.*, 2006; Noaksson *et al.*, 2001).

Histopathology is a valuable tool for assessment of chemical-induced alterations in gonads and is considered as an important endpoint in testing of

EDCs. For ovaries, specific endocrine-related diagnoses have been described, such as perifollicular hyperplasia and decreased vitellogenesis (decreased yolk formation), i.e. absent or smaller yolk granules than in the normal vitellogenic oocytes (OECD, 2009a). Other diagnoses, which may have various causes, are follicular atresia and stage changes. Examples of atretic follicles and oocytes with decreased vitellogenesis are shown in figure 1.

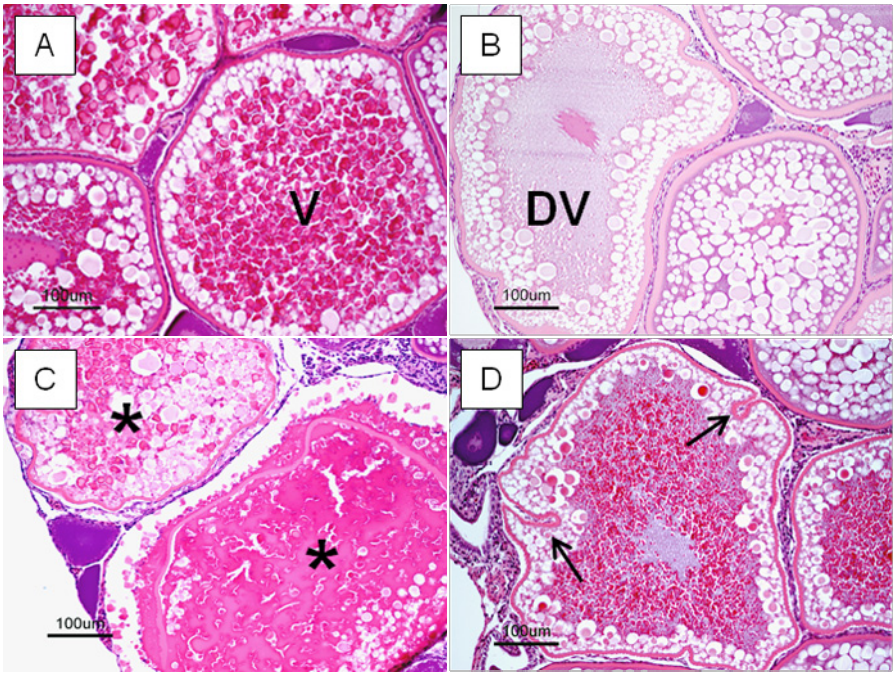

Figure 1. Light micrographs of ovaries from zebrafish exposed to control feed (A) or feed contaminated with 2,4,6-tribromophenol (B-D), showing different histopathological diagnoses. V = normal vitellogenic oocyte, DV = oocyte with decreased vitellogenesis, asterisk = atretic follicle, arrow = membrane folding. Haematoxylin-eosin staining.

Reduced ovarian maturity, i.e. fewer vitellogenic oocytes, has for instance been shown in zebrafish after exposure to PCBs (Örn *et al.*, 1998), various EDCs, such as estrogenic and androgenic (van der Ven *et al.*, 2003b), and to TCDD (King Heiden *et al.*, 2006). Decreased vitellogenesis has been observed after exposure of adult female fish to androgenic and aromatase inhibiting compounds, and has been interpreted as ineffective yolk formation or deposition (OECD, 2009a). Increased number of atretic follicles have for example been reported after exposure to EDCs, such as anti-estrogenic, estrogenic, androgenic and aromatase inhibiting compounds

(Van der Ven *et al.*, 2007; Ankley *et al.*, 2005; Pawlowski *et al.*, 2004; Ankley *et al.*, 2002; Van den Belt *et al.*, 2002).

1.6.3 Altered early life-stage development

In addition to effects on adult females, several effects on early life stages have been reported in Baltic Sea fish. Since the early 1970's, with a peak during the early 1990's Baltic salmon (*Salmo salar*) have suffered from high early life-stage mortality (the M74 syndrome), which has been related to low concentrations of thiamine (Amcoff *et al.*, 1998a; Amcoff *et al.*, 1998b). The origin of the M74 syndrome is still unknown. A connection between dioxin-like compounds and development of M74 has been suggested (Pesonen *et al.*, 1999; Lundström *et al.*, 1998; Vuorinen *et al.*, 1997; Norrgren *et al.*, 1993a), but the role of environmental pollutants is not clear. In a survey by Asplund *et al.* (1999), more than 100 halogenated phenol-type compounds, which may interfere with the hormone system in fish, were detected in Baltic salmon blood; however, no correlation to M74 could be established. In the southern part of the Baltic sea, reduced hatching rate in flounder (*Platichtys flesus*) has been correlated to PCBs (Von Westernhagen *et al.*, 1981), and malformed eelpout (*Zoarces viviparus*) larvae have been suggested to be related to degree of pollution at the sampling site (Gercken *et al.*, 2006). Moreover, perch living close to pulp and paper mill effluents have been shown to be affected by low recruitment, larval deformities and mortality (Sandström, 1994; Karås *et al.*, 1991). Low recruitment is also currently observed in perch and pike (*Esox lucius*) along the Baltic southeastern coast, most probably due to mortality in early larval stages (Ljunggren *et al.*, 2005); large scale changes in the pelagic ecosystem have been pointed out as a plausible cause.

In laboratory studies, reduced hatching has for example been reported in Japanese medaka (*Oryzias latipes*) after exposure to the synthetic estrogen 17α-ethinylestradiol (Tilton *et al.*, 2005), and delayed hatching in zebrafish embryos water exposed to BDE 47 (Lema *et al.*, 2007). Decreased larval survival has for instance been observed in offspring to zebrafish exposed to PCBs (Örn *et al.*, 1998) and TCDD (Heiden *et al.*, 2005). Typical TCDD-induced early life-stage toxicity was also observed in the study by Heiden *et al.* (2005), such as yolk sac, pericardial, and cranial edema, cardiac malformations, hemorrhage, and tail necrosis.

1.7 Brominated environmental chemicals

The brominated organic compounds that have received most attention over the past decades are those used as flame retardants; especially the PBDEs due to their similar structural and environmental properties with PCBs (Alaee *et al.*, 2003; Vos *et al.*, 2003). Besides man-made brominated organic compounds, more than 1 000 naturally occurring brominated organic compounds have been identified, for example in sponges, algae, bacteria and in marine worms (Gribble, 2000). One suggested function of natural brominated organic compounds is as a defense against predators. Natural brominated compounds have been suggested to bioaccumulate in predators in a similar way as those from anthropogenic sources, and have been detected throughout the aquatic food web, e.g. in fish and marine mammals (Vetter & Janussen, 2005; Tittlemier *et al.*, 2002; Vetter *et al.*, 2002; Whitfield *et al.*, 1998). Some of these brominated organic compounds with proposed natural origin are identical to those that originate from anthropogenic sources, e.g. the bromophenol 2,4,6-tribromophenol and brominated dioxins.

1.7.1 Brominated flame retardants

Brominated flame retardants (BFRs) are a heterogeneous group of more than 75 substances with different molecular structures and properties (Alaee *et al.*, 2003). They are used in flammable materials such as plastics, foams, paints, and textiles to increase their resistance to fire. BFRs are spread into the environment from production, use, and after disposal. Depending on their mode of incorporation into polymers, the BFRs can be divided into brominated monomers, additives and reactives. Additives are not chemically bound to the polymer (Hutzinger & Thoma, 1987) and may leach from products. Even reactives, which are covalently bound to the polymer, may leak out of products if not all of the reactive compound have polymerized (de Wit, 2002).

Many BFRs have been detected in wildlife and humans (Covaci *et al.*, 2006; Law *et al.*, 2006; Law *et al.*, 2003; Sjödin *et al.*, 2003; de Wit, 2002). Some of the most extensively used BFRs, such as the PBDEs and hexabromocyclododecane (HBCD), have low water solubility, bind to particles and sediments (de Wit, 2002), and bioaccumulate in fish (Haukås *et al.*, 2009; Eljarrat *et al.*, 2004; Kierkegaard *et al.*, 1999; Sellström *et al.*, 1998). Several BFRs have shown endocrine disrupting activity, both on the thyroid system and on estrogen- and androgen-mediated processes (Legler, 2008; Legler & Brouwer, 2003).

In fish, effects on reproduction and early life-stage development have been reported for tetrabromobisphenol A (TBBPA) (McCormick *et al.*, 2010; Kuiper *et al.*, 2007), 2,4,6-tribromophenol (TBP) (Deng *et al.*, 2010), HBCD (Deng *et al.*, 2010), BDE 47 (Lema *et al.*, 2007), and commercial PBDE mixtures (Timme-Laragy *et al.*, 2006). For most BFRs, however, little is known about effects on fish reproduction and early life-stage development.

1.7.2 Brominated dioxins

Brominated dioxins (polybrominated dibenzo-*p*-dioxins, PBDDs) are not intentionally produced, but are formed in the production of BFRs and during combustion of bromine containing waste (Wang & Chang-Chien, 2007; Sakai *et al.*, 2001; WHO, 1998). Moreover, several biogenic pathways have been proposed, e.g. formation via the environmentally abundant TBP (Haglund *et al.*, 2007). Debromination of PBDDs, e.g. tetraBDDs, have also been suggested (Haglund, 2010).

Recently, relatively high levels (ng to μg/g lipids) of PBDDs were detected in Baltic Sea biota, mainly in the productive coastal zone of the Baltic proper (Unger *et al.*, 2009; Malmvärn *et al.*, 2008; Haglund *et al.*, 2007; Malmvärn *et al.*, 2005). Concentrations of PBDDs in Baltic proper littoral fish and mussels were generally higher than polychlorinated dibenzo-*p*-dioxins (PCDDs) (Haglund *et al.*, 2007; Malmvärn *et al.*, 2005). In mussels, concentrations of PBDDs were found to increase over time, i.e. between the years 1995 and 2003, with large year-to-year variations (Haglund *et al.*, 2007). Among the PBDDs found in the Baltic Sea, di- and tri-substituted BDDs were most abundant (Haglund *et al.*, 2007). PBDDs have also been detected in sediments and biota from other parts of the world (Terauchi *et al.*, 2009; Fernandes *et al.*, 2008).

The structure of PBDDs is similar to the highly toxic PCDDs, and typical dioxin-like effects of PBDDs have been reported in studies with rodents (D'Silva *et al.*, 2004; Birnbaum *et al.*, 2003; Weber & Greim, 1997). In fish, *in vitro* induction of cytochrome P4501A (CYP1A) has been shown for several PBDDs (Olsman *et al.*, 2007), and *in vivo* CYP1A induction after exposure to commercial PBDE mixtures, known to contain PBDDs/polybrominated dibenzofurans (Kuiper *et al.*, 2006; Norrgren *et al.*, 1993b). Besides increased early life-stage mortality in rainbow trout (*Oncorhynchus mykiss*) exposed to 2,3,7,8-tetrabromodibenzo-*p*-dioxin (TBDD) and 2,3,7-trichlorodibenzo-*p*-dioxins (Hornung *et al.*, 1996a; Hornung *et al.*, 1996b), little is known about effects of PBDDs on fish reproduction and development. However, effects of PBDDs on fish health

and reproduction could be expected to be similar to those observed in fish exposed to the chlorinated dioxin, TCDD, e.g. reduced reproductive output, altered gonad morphology, and CYP1A induction (King Heiden *et al.*, 2006; Zodrow *et al.*, 2004; Wannemacher *et al.*, 1992).

Due to the high concentrations of PBDDs in the Baltic Sea biota, and due to the expected toxicity of the PBDDs, concern has been raised about their possible biological effects.

1.8 Zebrafish (*Danio rerio*) as a model species

The zebrafish is a small tropical cyprinid, whose natural habitats are mainly in river basins in north-eastern India, Pakistan, Nepal and Bangladesh (Spence *et al.*, 2008; Laale, 1977). In contrast to many fish species in temperate zones, the zebrafish have an opportunistic strategy, which is characterized by early sexual maturation, small body size, short generation time, frequent reproduction during an extended spawning season, and rapid larval growth (Hutchinson *et al.*, 2006). The lipid content in zebrafish eggs, i.e. approximately 3% of the wet weight (Nyholm *et al.*, 2009), is similar to that of some species native in Swedish waters, such as herring (*Clupea harengus*), and cod (*Gadus morhua*) (Petersen & Kristensen, 1998), but lower than that of salmon, i.e. approximately 7-9% of the wet weight (Asplund *et al.*, 1999; Hayes & Ross, 1937).

The zebrafish is one of four species considered for testing of reproductive toxicity. Some of the advantages of using zebrafish in studies of reproductive output and early life-stage development are that offspring can be produced continuously and at high numbers, embryonic development is rapid, and that the chorion is transparent, which enables studies of phenotypic changes during the embryo development. The zebrafish has long been an important model organism in genetics, toxicology and developmental biology, and there is much information on developmental and genetic mechanisms. The genome of zebrafish is sequenced (http://zfin.org), and a broad variety of biomarkers are possible to measure, such as induction of Vtg and CYP1A (Jönsson *et al.*, 2009; Holbech *et al.*, 2001). Regarding sensitivity of biomarkers, Vtg induction, which is a biomarker of estrogenic disruption, in zebrafish seems to be similarly sensitive as that in other fish species (Hutchinson *et al.*, 2006), whereas CYP1A induction have been reported to be less sensitive compared to for instance the rainbow trout (Jönsson *et al.*, 2009).

2 Aims of the thesis

The general aim was to investigate whether dietary exposure to brominated environmental chemicals, in mixtures or individually, affects reproductive health and early life-stage development in zebrafish.

The specific aims were:

- To investigate whether a set of 11 structurally diverse BFRs are maternally transferred to offspring, and whether these BFRs affect reproduction and early life-stage development in zebrafish.

- To study reproductive effects and early life-stage development in zebrafish after exposure to TBP, the extent to which TBP was metabolized to 2,4,6-tribromoanisole (TBA), and possible transfer of TBP and TBA to offspring.

- To study retention and maternal transfer of PBDDs in zebrafish and effects on reproduction and early life-stage development. Moreover to study whether PBDDs can activate gene transcription through the AHR pathway in zebrafish.

3 Materials and Methods

This thesis is based on four experiments approved by the local ethical committee. Details on the material and methods used are presented in the individual paper I-IV. Here, materials and methods are presented in a summarized form with focus on experimental designs and core endpoints.

3.1 Chemicals

A set of 10 BFRs with a large structural variation has previously been suggested to be used in assessments of BFRs persistency, bioaccumulation and toxicity properties and in the development of structure-activity relation models for prediction of other non-tested BFRs (Andersson *et al.*, 2006). These were selected to cover the range of physico-chemical properties of a larger group of 65 brominated organic compounds produced for use as flame retardants. Moreover, production volumes, environmental relevance, and properties that facilitate chemical analysis and experimental handling were considered in the selection. The BFRs tested in this thesis are listed in table 1. These include the 10 BFRs selected by Andersson *et al.* (2006), with the addition of an 11th BFR, i.e. tetrabromobisphenol A 2,3-dibromopropyl ether (TBBPA DBPE).

Table 1. *The brominated flame retardants (BFRs) studied, with chemical names and abbreviations*

Chemical name	Abbreviation
2-bromostyrene	BrSty
2,4,6-tribromophenol	TBP
1,2,-dibromo-4-(1,2-dibromoethyl)cyclohexane	TBECH
2,4,4'-tribromodiphenyl ether	BDE 28
tetrabromobisphenol A 2-hydroxyethyl ether	TBBPA OHEE
tetrabromobisphenol A	TBBPA
hexabromobenzene	HxBrBz
hexabromocyclododecane	HBCD
2,2',3,4,4',5',6-heptabromodiphenyl ether	BDE 183
tetrabromobisphenol A 2,3-dibromopropyl ether	TBBPA DBPE
decabromodiphenyl ether	BDE 209

The PBDDs tested are listed in table 2. These include TBDD (positive control) and a mixture of twelve PBDDs (Baltic Sea mixture). The mixture was designed to reflect relative concentrations of the PBDDs found in Baltic Sea biota. TBDD, which has been detected in only few fish samples from the Baltic Sea at very low concentrations (Haglund *et al.*, 2007), was tested due to its high potency in other test systems.

Table 2. *The brominated dioxins (PBDDs) studied, with chemical names and abbreviations*

Chemical name	Abbreviation
Baltic Sea mixture	
1- monobromodibenzo-*p*-dioxin	1-MBDD
2,7/2,8- dibromodibenzo-*p*-dioxin	27/28-DBDD
1,3,7- tribromodibenzo-*p*-dioxin	137-TrBDD
1,3,8- tribromodibenzo-*p*-dioxin	138-TrBDD
2,3,7- tribromodibenzo-*p*-dioxin	237-TrBDD
1,3,6,8- tetrabromodibenzo-*p*-dioxin	1368-TeBDD
1,3,7,9- tetrabromodibenzo-*p*-dioxin	1379-TeBDD
1,2,4,7/1,2,4,8- tetrabromodibenzo-*p*-dioxin	1247/1248-TeBDD
1,2,3,7- tetrabromodibenzo-*p*-dioxin	1237-TeBDD
1,2,3,8- tetrabromodibenzo-*p*-dioxin	1238-TeBDD
Positive control	
2,3,7,8-tetrabromodibenzo-*p*-dioxin	TBDD

BDE 28 is present at low concentrations in the commercial pentaBDE-mix Bromkal 70-5DE (Sjödin *et al.*, 1998). BDE 28 is widespread in the environment and has been detected in e.g. sewage sludge, fish, and humans (Covaci *et al.*, 2008; Sjödin *et al.*, 2008; Knoth *et al.*, 2007; Vives *et al.*, 2004).

BDE 183 is the major component of the commercial octaBDE-mix Bromkal 79-8DE (Korytár *et al.*, 2005). BDE 183 is ubiquitously found in the environment, for example in sewage sludge, fish, birds of prey, and humans (Antignac *et al.*, 2009; Chen *et al.*, 2007; Knoth *et al.*, 2007; Rice *et al.*, 2002).

The most common technical product among the PBDEs is the decaBDE (de Wit 2002), which consist almost exclusively of **BDE 209**. DecaBDE is listed as a high production volume chemical (HPVC) within OECD and EU, i.e. the production exceeds 1 000 tonnes per year in at least one OECD member country (listed in year 2004), or that it is placed on the European market in volumes that exceeds 1 000 tonnes per distributor and year (http://www.oecd.org, http://ecb.jrc.ec.europa.eu/esis). Like BDE 28 and 183, BDE 209 has been detected in various environmental compartments, such as in sewage sludge, fish, birds of prey, and humans (Antignac *et al.*, 2009; Shaw *et al.*, 2009; Chen *et al.*, 2007; Knoth *et al.*, 2007; Öberg *et al.*, 2002).

HBCD technical products consist of three diasteroeisomers, α, β and γ, in different proportions. HBCD is used as an additive BFR (Alaee *et al.*, 2003). In 2001, HBCD was the second highest-volume BFR used in Europe (Alaee *et al.*, 2003), and were listed as an HPVC both by the OECD and EU. HBCD is a ubiquitous contaminant, which has been found in high concentrations in sediments and fish downstream of production sites, industrial users, and downstream heavy industrialised areas (Morris *et al.*, 2004; Remberger *et al.*, 2004; Sellström *et al.*, 1998). Biomagnification of HBCD has been suggested in aquatic food webs (Law *et al.*, 2006; Morris *et al.*, 2004; Tomy *et al.*, 2004).

TBBPA is produced in large volumes, e.g. an OECD and EU HPVC, and is used both as a reactive and an additive BFR (WHO, 1995). In the environment, TBBPA has for example been detected in sediments, sewage sludge, fish, and human milk (Cariou *et al.*, 2008; Morris *et al.*, 2004; Asplund *et al.*, 1999; Sellström & Jansson, 1995).

TBBPA OHEE is a derivative from TBBPA and is used as an additive BFR (WHO 1995). Within EU, between 10 and 1000 tonnes are placed on the market per distributor and year (http://ecb.jrc.ec.europa.eu/esis).

TBBPA DBPE is another derivate of TBBPA used as an additive BFR (WHO, 1995), and listed as an OECD HPVC.

TBP is used as a reactive BFR, an intermediate in the production of other BFRs (OECD, 2004; WHO, 1995), and as a fungicide (Savory *et al.*, 1970). The global production of TBP was 9,500 ton in 2001 (OECD, 2004) and TBP is listed as a HVPC within EU and OECD. TBP is also formed by photolytic degradation of TBBPA (Eriksson & Jakobsson, 1998), and natural production of TBP has been proposed in marine organisms, such as marine worms and algae (Gribble, 2000; Whitfield *et al.*, 1999; Chen *et al.*, 1991; Higa *et al.*, 1980; Weber & Ernst, 1978). TBP has been detected in various environmental compartments; for example, in sewage sludge (Öberg *et al.*, 2002), marine and fresh waters (Sim *et al.*, 2009; Reineke *et al.*, 2006; IUCLID, 2003), sediments (Sim *et al.*, 2009; Tolosa *et al.*, 1991; Watanabe *et al.*, 1985), fish (Whitfield *et al.*, 1998), marine mammals (Vetter & Janussen, 2005), and in humans (Hovander *et al.*, 2002; Thomsen *et al.*, 2002).

TBECH commercial mixtures are produced as additive flame retardants and consists mainly of two isomers, α and β (Arsenault *et al.*, 2008). TBECH occurs in different environmental compartments, e.g. in industrial waste water (Santillo *et al.*, 1997), arctic mammals (Tomy *et al.*, 2007), and in eggs from fish eating birds (Gauthier *et al.*, 2008).

HxBrBz was one of the most used brominated flame retardants in Japan during the 1980's (Yamaguchi *et al.*, 1988). The current production is low. HxBrBz may be formed during the natural debromination of highly brominated BDEs (Buser, 1986). In fish, zero or minimal accumulation of HxBrBz from both from water and food has been reported (Oliver & Niimi, 1985; Zitko & Hutzinger, 1976). Metabolism of HxBrBz has been suggested to partly explain the low accumulation from feed in fish (Nyholm *et al.*, 2009).

BrSty is a brominated monomer (Andersson *et al.*, 2006) used to give flame retardant properties to polymers. The production and environmental occurrence of BrSty is low.

3.2 Feed preparation

Experimental feed was prepared by adding the test chemicals dissolved in ethanol to freeze-dried chironomids (Nutrafin™, paper I and II, Nutrafin® basix, paper III and IV). Chironomids treated with ethanol only was used as control feed (paper I-IV). In paper I and II, the 11 BFRs (Table 1) were mixed in equal molar amounts and added to chironomids. In paper III, feed

containing TBP alone was prepared. In paper IV, feed was prepared containing TBDD alone, or a mixture of 12 PBDDs (Baltic Sea mixture, Table 2). After adding the test chemicals to the feed and mixing thoroughly, the ethanol was evaporated. The prepared feed was stored in dark to avoid photo degradation (paper I-IV).

3.3 Animals and general experimental design

Adult zebrafish were bought from a local fish supplier and acclimatized for at least three weeks in the laboratory prior to experiments. During acclimatization and throughout the experiment, fish and offspring were kept in aerated standardized water (ISO, 1996) prepared from deionized water with the addition of $CaCl_2 \times 2H_2O$ (117.6 mg/L), $MgSO_4 \times 7H_2O$ (49.3 mg/L), $NaHCO_3$ (25.9 mg/L), and KCl (2.3 mg/L). The temperature was 25 ± 1°C and the photoperiod 12/12 h. Water quality characteristics were in accordance with OECD guidelines (OECD, 2008). For breeding trials, fish were held in stainless steel net cages within glass aquaria to separate the fish from their eggs to avoid predation. Fish were fed untreated chironomids (embryo toxicity test, paper II) or experimental feed (reproductive assays, paper I-IV). All fish were also fed brine shrimp (*Artemia*, FlatPak™) as additional nutrition. Breeding started at the onset of light and eggs were collected 30 min later from the bottom of the aquaria by siphoning. Eggs that had reached the four-cell stage were considered fertilized (Schulte & Nagel, 1994). Water was renewed semi-statically, i.e. one-third (paper I and II) or two-thirds (paper III and IV) of the water was exchanged six (paper III) or seven (paper I, II, and IV) days per week. Feces and eggs were removed from the bottom of the aquaria at each water exchange. Prior to sampling, fish were euthanized in MS222 and killed by decapitation.

3.4 Reproductive assays (paper I-IV)

The reproductive assays used in this thesis are based on the 21-day OECD guideline Fish Short Term Reproduction Assay (FSTRA) (OECD, 2009b), which primarily was designed to screen for effects of EDCs (e.g. Vtg induction), but also for other reprotoxic effects (e.g. fecundity). Modifications of the FSTRA guideline were made in this thesis, i.e. use of additional endpoints, prolonged exposure periods, and exposure via the diet. Endpoints such as fertilization success, and offspring viability, i.e. embryo development, hatching success, and survival, were added to extend the evaluation of reprotoxic effects and to investigate effects of maternal transfer.

Prolonged exposure periods (paper I, III and IV) were used to be able to reach steady state of the compounds. Dietary exposure was used due to the high hydrophobicity of many of the compounds tested (paper II and IV), and to investigate effects of the oral route (paper III).

Adult male and female zebrafish were exposed to control feed or feed spiked with the brominated compounds at about 2% of their body weight per day. To ensure that all feed was consumed and to minimize the risk of water contamination, fish were fed half of the dose in the morning and the other half in the afternoon. In the studies that examined reproductive effects (paper II-IV), the exposure period was preceded by a pre-exposure period, which was between one and three weeks, during which all groups were fed control feed. The different exposure periods and other specific conditions in each of the studies are described below.

Paper I and II

The experiments presented in paper I and II were run in parallel. Fish were exposed to control feed or feed contaminated with the BFR mixture at nominal concentrations of 10 and 100 nmol (paper I) or 1, 10 and 100 (paper II) nmol of each compound per g dry weight. In paper I, the exposure period was 6 weeks. Each dose group consisted of 46 fish (approximately 23 males and 23 females) at start of exposure. No replicates were used. In paper II, the exposure period was 3 weeks. Each aquarium contained 12 adult zebrafish (approximately 8 males and 4 females) and two replicate aquaria were used per concentration.

Paper III

Fish were exposed for 6 weeks to control feed or feed contaminated with TBP at nominal concentrations of 33, 330, 3300 μg TBP/g dry weight (0.1, 1, 10 μmol/g). Each aquarium had nine fish (at least three females) and four replicate aquaria were used per concentration.

Paper IV

Fish were exposed for 9 weeks to control feed or feed contaminated with TBDD (3, 30, or 300 ng/g dry weight, nominal concentrations) or to the Baltic Sea mixture (Table 2), which was prepared in three dose levels (se paper IV), at concentrations reflecting relative concentrations of the PBDDs found in Baltic Sea biota. Each aquarium had 13 fish (at least 6 females), and 4 replicate aquaria were used per concentration.

3.5 Embryo toxicity test (paper II)

This test is based on an OECD draft guideline (OECD, 2006). Each of the 11 BFRs (Table 1) was dissolved in DMSO and tested individually in six concentrations (spaced by a constant factor of 2.0-2.2) based on range finding tests and solubility in DMSO. DMSO content in all treatments, including control (standardized water) was 0.1%. Eggs from unexposed adult male and female zebrafish were collected, examined under a stereo microscope, and transferred into test solutions within 1 h post fertilization (hpf). For each treatment, 24 fertilized eggs were placed individually in 96-well plates, one egg in each well, with 250 μL test solution.

In parallel to the embryo toxicity test, 96-well plates without eggs, but with test solutions, were set up to analyze the BFR content in the water phase. Water samples for chemical analysis were first taken directly from the glass beaker after preparing the test solution (before adding the solution to the plates), and then after 24 h and 144 h on the plates. All water samples were stored in dark at -20°C until chemical analysis.

3.6 Accumulation from feed and maternal transfer

Exposed adult females and newly laid eggs were sampled to investigate how much of the chemicals spiked to feed that was retained in females and transferred to offspring. In all studies, adult fish were sampled 24 h post feeding and eggs were rinsed thoroughly in standardized water at sampling. Excess water was removed by using a Pasteur pipette. All samples were stored at -20°C until chemical analysis of the different brominated compounds. In paper I, adult females and eggs were sampled regularly during the exposure period for chemical analysis of the BFRs. In paper III, eggs and newly hatched fry were collected every second week during parental exposure, and adults at the end of exposure, for chemical analysis of TBP and the metabolite TBA. In paper IV, eggs were collected during week 5-9 of parental exposure, and fish at the end of exposure, for chemical analysis of PBDDs.

The analysis of brominated chemicals in feed, fish, eggs, and fry, is described in detail in paper I, III and IV. In brief, samples were homogenized and internal standards were added prior to extraction to compensate for possible losses. Extraction, cleanup and analysis by gas chromatography was done as described in Nyholm *et al.* (2009).

The analysis of BFRs in water is described in detail in paper II. In brief, internal standards were added to the water samples. The organic phase was

extracted, and a recovery standard was added prior to analysis by gas chromatography.

3.7 Reproductive output (paper II-IV)

Reproductive endpoints studied were fecundity, i.e. eggs per female and day, spawning success, i.e. percentage of days when >10 eggs were produced, and fertilization success, i.e. percentage of embryos that had reached the four-cell stage. To calculate the fertilization success, a sample of newly laid eggs (30-100) from each aquarium were randomly selected and examined under a stereo microscope. In paper II and III, eggs were monitored for fertilization success within approximately 2 hpf. Coagulated eggs and eggs with less than four cells were counted as unfertilized. Due to the large experiment in paper IV, the eggs were monitored later (at approximately 5 hpf) and number of coagulated eggs, eggs with less than four cells, or with asymmetrical cells were counted to give a proportion of unfertilized and abnormal offspring at 5 hpf. In all studies, spawning fitness and fertilization success were monitored during one week prior to start of experiment to check the reproductive fitness of the spawning groups.

3.8 Early life-stage development

Zebrafish early life-stage development was monitored until 144 hpf, which is the approximate time when the larvae start feeding actively (OECD, 2006).

3.8.1 Maternal exposure (paper II-IV)

In the reproductive assays, eggs from exposed parental fish were collected for studies of early life-stage development during pre-exposure and at the end of the adult exposure period (paper II-IV).

Table 3. Endpoints studied in zebrafish embryos and larvae at different time points post fertilization, and criteria for each parameter

Endpoint	Criteria	24 h	48 h	144 h
Coagulation[1]	Partial or total coagulation of embryos, yes/no	x	x	x
Tail extension[3]	Extension of tail ranked 1-3:	x		
	1=normal, fully extended			
	2= shorter, but detached from yolk sac			
	3= not detached from yolk sac			
Movement pattern[3]	Movement pattern within 30s ranked 1-3:	x		
	1=normal full movements			
	2=movements in tail only			
	3=no movements			
Movements/min[2]	Number of movements/min. All movements were counted: both full rotations and movements only in the tail	x		
Developmental stage[3,4]	Developmental stage ranked 1-4:	x		
	1=normal 24 h development			
	2= 16.5-21.5 h development			
	3= 13-15.5 h development			
	4= 5.5-9 h development, gastrula			
Circulation[1]	Flow of blood cells visible in the caudal artery in tail, yes/no		x	
Edema[1]	Edema, yes/no		x	
Pigmentation[1]	Dark eyes and dark pigmentation spots on body, yes/no		x	
Heart rate[2]	Time for 30 heart beats registered and converted to beats/minute		x	
Heart beat[1]	Heart beats registered 15 s from observation start, yes/no		x	
Hatching success[1]	Hatched embryos, yes/no			x
Sidewise position[1]	Larvae in a sidewise position at the bottom of the well, yes/no			x

[1] Categorical endpoint (yes or no)

[2] Continuous endpoint

[3] Ordinal endpoint (ranked values)

[4] According to Kimmel *et al.* (1995)

In paper III, eggs were collected every second week of adult exposure. Eggs were rinsed thoroughly in standardized water, and examined under a stereo microscope. Fertilized eggs were selected and then either transferred to Petri dishes (20 eggs in 50 mL standardized water) (paper II), or individually transferred to 96-well plates (on egg in 250 μL standardized water) (paper III and IV). From each spawning aquarium, 40 (paper II), 12 (paper III) or 16 (paper IV) eggs were studied. In paper II, numbers of hatched and dead individuals were recorded two times per day. In paper III, embryos were examined for different endpoints under a stereo microscope at 24, 48 and 144 hpf (Table 3). In paper IV, numbers of coagulated eggs, eggs with less than four cells, or with asymmetrical cells were counted to give a proportion of unfertilized and abnormal offspring at 5 hpf (as described above). At 144 hpf, larvae were examined under a stereo microscope for yolk-sac edema, spine or cranial deformities, spontaneous movement, hatching, and mortality (paper IV). Mortality was defined as coagulation or lack of heart beat (paper III and IV).

3.8.2 Water exposure (paper II)

In the embryo toxicity test, eggs from unexposed parental zebrafish were collected and exposed to BFRs via water as described above. Embryos were examined for different endpoints (Table 3) under a stereo microscope at 24, 48 and 144 hpf. Mortality was defined as lack of heart beat or coagulation.

3.9 Histopathology and immunohistochemistry

Samples for histological evaluation were fixed in phosphate-buffered formalin, dehydrated, and embedded in paraffin blocks. Sections were cut from three different levels of each gonad, i.e. from the dorsal to the ventral side, stained with haematoxylin-eosin and examined by light microscopy. An initial evaluation of control and high-dose exposed groups, to identify treatment related changes, was followed by a more thorough investigation on coded slides. Ovarian maturity was evaluated with respect to oocytes in the following stages: (1) perinucleolar (primary growth stage IB), (2) cortical alveolar and (3) vitellogenic oocytes (Selman *et al.*, 1993). The proportion of different oocyte maturation stages (paper III and IV), atretic follicles (paper II-IV), membrane folding (paper III), and oocytes with decreased vitellogenesis (paper III) was calculated either by counting the number of oocytes/follicles in the different categories per microscopic field (paper II and III) or by using stereological point counting (paper IV). In males, testes were screened for effects on maturation and degeneration. Presence of

spermatozoa (paper II and III) and number of spermatid cysts per microscopic field was counted (paper III).

In paper III, immunohistochemistry was performed to confirm the changes in vitellogenin content indicated on haematoxylin-eosin stained sections. In brief, zebrafish-specific rabbit anti-lipovitellin polyclonal antiserum (Holbech et al., 2001) was diluted in phosphate-buffered saline and incubated at room temperature on routinely pre-treated sections. As a negative control, the primary antibodies were replaced with rabbit IgG. Immunoreactions were visualized using the Dako REAL EnVision™ Detection system. Sections were incubated with EnVision/HRP, Rabbit/Mouse for 30 min and diaminobenzidine chromogen for 3 min. Sections were then counterstained with haematoxylin, dehydrated, and mounted.

3.10 Vitellogenin (paper III)

Vtg was measured in adult male and female fish at the end of the exposure period by methods described in paper III. In brief, head and tail from individual adult zebrafish were homogenized in a buffer. The homogenate was centrifuged and the supernatant was instantly frozen in liquid nitrogen and stored at -70°C. Vtg concentrations were determined by a method based on a direct non-competitive sandwich enzyme-linked immunosorbent assay (ELISA) previously described by Holbech *et al.* (2001).

3.11 EROD and GR (paper IV)

Hepatic ethoxyresorufin-*O*-deethylase (EROD) activity and glutathione reductase (GR) activity were measured in adult male and female fish at the end of the exposure period by methods described in paper IV. In brief, two zebrafish liver samples of the same sex and exposure group were pooled and sonicated in homogenization buffer. The homogenate was centrifuged and the supernatant was instantly frozen in liquid nitrogen and stored at -80°C until analysis. EROD activity was measured as described by Förlin *et al.* (1994), and GR activity was measured as described by Cribb *et al.* (1989). Protein content was determined according to a method described by Lowry *et al.* (1951).

3.12 Gene expression (paper IV)

Hepatic expression of a number of potential target genes, selected based on their known responsiveness to AHR agonists, were studied to investigate whether PBDDs can activate gene transcription through the AHR pathway in zebrafish. Expression of inducible genes involved in adaptive responses to intracellular stress was also studied to investigate potential stress effects of PBDDs. These biomarkers were measured in adult male and female fish at the end of the exposure period by a method described in paper IV. In brief, total RNA was isolated from liver samples of individual zebrafish and expression of mRNA of the genes of interest was measured using SYBR green real-time reverse transcription polymerase chain reaction (real-time RT PCR). The measured mRNA was normalized against total RNA content and was then quantified using a standard curve of known concentrations.

3.13 Statistics

To test for differences between groups at the end of the exposure period, a one-way analysis of variance (ANOVA) followed by Dunnet's post hoc test (Minitab 15) or Bonferroni's multiple comparison test (GraphPad Prism 5.01) was used to analyze data on body weight (paper II and III), hepatic- and somatic index (paper II), histopathological endpoints (paper II-IV), Vtg concentration (paper III), reproductive output (paper III), and continuous and categorical data on offspring early life-stage development (paper III and IV). Ordinal data on offspring early life-stage development in paper III were analyzed using Kruskal-Wallis test followed by Mann-Whitney U test (Minitab 15).

To test for treatment- and time-related effects, a two-way ANOVA repeated measurements followed by Bonferroni's multiple comparison test, with treatment and time as factors, were used to analyze body weight (paper IV), and reproductive output (paper II and IV) (GraphPad Prism 5.01).

To test for treatment- and gender-related effects (paper IV), data on gene expression, EROD and GR activities were analyzed using a two-way ANOVA followed by Bonferroni's multiple comparison test with treatment and sex as factors (GraphPad Prism 5.01).

In the embryo toxicity test (paper II), continuous endpoints were analyzed using one-way ANOVA followed by Dunnet's post hoc test (Minitab 15), ordinal endpoints using Kruskal-Wallis test followed by Mann-Whitney U test (Minitab 15), and categorical endpoints using Fisher's Exact Test followed by Bonferroni adjustment of p values (StatView 5.0.1).

The statistical analyses were made on replicate mean values, except for the gene expression data, which were analyzed using 5-8 individuals per group, randomly selected from the four replicates. Transformation of the data was performed when necessary to meet the ANOVA requirements. The significance level was set at 95% ($p \leq 0.05$).

4 Results and discussion

Results are presented in detail in the paper I-IV included in this thesis. In this section, the main findings are presented and discussed.

4.1 Paper I and II

The first two studies investigated whether a set of 11 structurally diverse BFRs are maternally transferred to offspring, and whether these BFRs affects reproduction and embryo development in zebrafish.

After dietary exposure of adult zebrafish to a mixture of the selected BFRs, eight of the BFRs were detected in females and eggs (paper I). In female fish, highest concentrations were found for BDE 28, HBCD, and BDE 183, followed by TBECH and TBBPA DBPE. These BFRs all had log K_{ow} values around six or higher. However, no linear relationship was observed between log K_{ow} values of the BFRs and the levels in fish. Several tentative metabolites were also detected in fish and eggs, e.g. potential debromination products of BDE 183 and BDE 209, and a potential metabolite of TBP. The low concentrations of TBBPA and HxBrBz are in accordance with previous findings (Kuiper *et al.*, 2007; Zitko & Hutzinger, 1976). Exposure to the BFR mixture significantly increased the number of atretic follicles in female zebrafish. This might be explained by the documented endocrine disrupting potential of several of the BFRs, e.g. estrogenic (Hamers *et al.*, 2006; Meerts *et al.*, 2001; Kester *et al.*, 2000), anti-estrogenic (Canton *et al.*, 2005), androgenic (Larsson *et al.*, 2006), and anti-androgenic (Hamers *et al.*, 2006). Exposure of adult fish to EDCs have previously resulted in increased ovarian atresia (Van der Ven *et al.*, 2007; Ankley *et al.*, 2005; Pawlowski *et al.*, 2004; Ankley *et al.*, 2002; Van den Belt *et al.*, 2002). Atretic follicles may also result from reduced spawning, however, no significant reduction in reproductive output was observed. In

contrast to females, no effects were observed on male gonad morphology. Morphological effects on females but not on males have previously been described after exposure to BFRs (Kuiper *et al.*, 2006; Zeller & Kirsch, 1969). A possible explanation could be that the BFRs bind to lipoproteins important for oocyte growth, and thus are incorporated in the eggs during vitellogenesis. The mechanisms of the maternal transfer were outside the scope of this thesis, but the fact that the lipid adjusted concentrations of the BFRs were higher in eggs than in adult female fish (paper I), may support this hypothesis.

In eggs, highest concentrations were found for HBCD, BDE 28, followed by BDE 183 and TBBPA DBPE. Concentrations in eggs, relative to concentrations in females, were highest for the BFRs with highest log K_{ow}, i.e. lipid normalized egg/fish concentration ratios were significantly higher than 1.0 for BDE 183, TBBPA DBPE, and BDE 209 (log K_{ow} 8-12). This indicates that highly hydrophobic BFRs are more efficiently transferred than less hydrophobic BFRs. Hatching success was significantly reduced in offspring from fish exposed to the BFR mixture high dose (paper II), i.e. 20% of the embryos were unhatched, but alive, at 144 hpf. Factors that may result in delayed hatching are, for example, retarded development or inability of embryos to break the chorion. Delayed hatching has been observed in zebrafish exposed during the embryonic period via water to BDE 47 (Lema *et al.*, 2007). Among the BFRs included in this thesis, reduced hatching has previously been observed for TBBPA after exposure of parental fish followed by water exposure of the offspring (Kuiper *et al.*, 2007). TBBPA was detected in eggs from fish exposed to the BFR mixture, but in relatively low concentrations (paper I).

All BFRs detected in females and eggs have shown endocrine disrupting activity *in vitro* (Khalaf *et al.*, 2009; Ding *et al.*, 2007; Hamers *et al.*, 2006; Larsson *et al.*, 2006; Schriks *et al.*, 2006; Canton *et al.*, 2005; Yamada-Okabe *et al.*, 2005; Legler *et al.*, 2002; Kester *et al.*, 2000; Meerts *et al.*, 2000), except for BDE 183, which have shown weak AHR-agonistic potency (Hamers *et al.*, 2006). Further studies are needed to reveal relative contribution of the BFRs to the observed effects and to investigate possible interactions between the BFRs. In the reproductive assay (paper II), fish were exposed to the BFR mixture for three weeks, which is the standard in the FSTRA guideline (OECD, 2009b). In male zebrafish exposed to the BFR mixture, reported by Nyholm *et al.* (2009), several of the BFRs did not reach steady-state concentrations within three weeks. Thus, a prolonged exposure period in the reproduction assay (paper II) might have revealed effects at lower, and more environmentally realistic, concentrations.

In the embryo toxicity test using water exposure (paper II), 8 of the 11 individually tested BFRs, were detected in the water phase. Of these eight, only TBP, TBPPA, TBECH, and TBBPA OHEE significantly affected zebrafish embryo development, and were thus able to pass the chorion and reach the embryo. For the more hydrophobic compounds present in the water, such as HxBrBz and BDE 183, the barrier function of the chorion (Braunbeck *et al.*, 2005) might partly explain the lack of effect on embryo development. The most hydrophobic BFRs were not detected in the water and could not be adequately assessed by this embryo toxicity test.

For TBP and TBBPA, the results from paper II confirmed previous findings on early life-stage effects, such as edema, decreased heart rate, decreased hatching and survival (McCormick *et al.*, 2010; Kuiper *et al.*, 2007; Kammann *et al.*, 2006). However, a dose-related delay in embryo development, and changed movement behavior, i.e. movements only in the tail, was observed after exposure to TBP. TBP has previously been shown to inhibit development in copepods (Wollenberger *et al.*, 2005), and to reduce growth of zebrafish larvae (Deng *et al.*, 2010). For TBBPA, TBECH, and TBBPA OHEE, alterations in movement behavior were observed, similar to that of TBP, and alterations in tail formation, similar to that previously observed for TBBPA (McCormick *et al.*, 2010). Exposure to all four compounds reduced the hatching success, i.e. embryos died before hatching, at different concentrations. There is no information available on effects of TBECH and TBBPA OHEE on fish early life-stages, however, both TBBPA OHEE and TBECH affects larval development in the copepod *Nitocra spinipes* (Breitholtz *et al.*, 2008). For TBECH and TBBPA OHEE, there is no data available on environmental water concentrations. For TBP and TBBPA, lowest observed effect concentrations (LOEC) were above reported environmental concentrations. In the study by Breitholtz *et al.* (2008), in which ten of the BFRs in the present thesis were tested, it was shown that low concentrations of individual BFRs at no observed effect concentrations (NOEC) can cause toxicity if exposed in mixtures. Thus, it is possible that the BFRs could cause effects at lower concentrations also in fish if exposed in a mixture.

Overall, the results from the first two studies (paper I and II) indicated that BFRs with a large variation in molecular structure are transferred from females to eggs, and affects zebrafish reproductive physiology and early life-stage development. Early-life stages were affected both via parental and water exposure.

4.2 Paper III

The third study investigated whether dietary exposure of adult zebrafish to TBP, one of the BFRs detected in females and eggs in paper I and II, affected reproduction and offspring early life-stage development. Concentrations of TBP in wild marine fish has been related to intake via feed (Chung *et al.*, 2003; Whitfield *et al.*, 1998; Whitfield *et al.*, 1997; Boyle *et al.*, 1992), but the relevance of the dietary exposure for fish health is not known.

Exposure of adult zebrafish to feed contaminated with TBP significantly reduced the fertilization success. TBP has previously been shown to reduce *in vitro* fertilization success in sea urchin (*Psammechinus miliaris*) (Schäfer *et al.*, 2009). In a study by Deng *et al.* (2010), reduced fecundity was observed in zebrafish exposed to TBP via water, but reduced fertility in fish after TBP exposure has not previously been described.

Altered gonad morphology was observed in zebrafish dietary exposed to TBP, with most pronounces effects in females. The changes in ovarian morphology were characterized by increased number of atretic follicles, predominantly in the vitellogenic stage, and by increased numbers of oocytes with decreased vitellogenesis (yolk formation). The yolk serves as nutrition for the developing larvae and sufficient amount of yolk deposited is therefore essential for growth and survival (Mommsen & Walsh, 1988). If oocytes with decreased vitellogenesis are ovulated and fertilized, growth and survival of offspring may be affected.

The altered ovarian morphology observed in females from the present study was accompanied by increased levels of circulating Vtg. It is known that Vtg that is not taken up by oocytes, can be found in high concentrations in the circulation (Van der Ven *et al.*, 2003a; Mommsen & Walsh, 1988). The increased atresia, almost exclusively in vitellogenic stage oocytes, and the decreased yolk formation in vitellogenic oocytes might be a result of lowered incorporation of Vtg in oocytes, and thus explain the increased circulatory Vtg levels observed.

Accumulation in adult zebrafish of TBP from the feed was low, which confirmed the results in paper I. This study also confirmed the finding in paper I that embryos can be exposed to TBP via maternal transfer. Moreover, TBA was detected in females and eggs, but not in the feed, which supports the hypothesis in paper I that TBP may be metabolized to TBA. Although both TBP and TBA were detected in eggs and fry, offspring early life-stage development was not significantly affected. The more pronounced effects previously observed in offspring from zebrafish exposed to waterborne TBP (Deng *et al.*, 2010), and the biotransformation of TBP

to the metabolite TBA in the dietary exposed zebrafish in the present thesis, may suggest that water exposure to TBP is more effective. The difference in response in relation to exposure route has been studied by Pickford *et al.* (2003), showing that a hydrophobic chemical, i.e. 4-*tert*-nonylphenol, for which both exposure via the gill and the GI may be ecologically relevant, has a higher estrogenic potential when fish are exposed via the water compared with exposure via the diet.

Overall, the results from paper III show that TBP exposed via feed may interfere with reproduction in fish. Effects were seen at environmentally realistic concentrations, i.e. at concentrations found in marine organisms that serves as diet of wild fish (Whitfield *et al.*, 1996), however, further studies are needed to investigate effects of TBP on ecologically relevant marine species.

4.3 Paper IV

The fourth study investigated effects of brominated dioxins in adult zebrafish, as well as maternal transfer and effects on offspring. Adult zebrafish were exposed to TBDD or to a mixture of PBDDs (Baltic Sea mixture), which was designed to reflect relative concentrations found in Baltic Sea biota.

All PBDDs spiked to the feed were detected in female fish and in eggs, but the biological significance of the maternal transfer was low. Early life-stage mortality has previously been shown in rainbow trout after egg injections of TBDD and 237-TrBDD (Hornung *et al.*, 1996a; Hornung *et al.*, 1996b); however, effects were seen at egg concentrations higher than in the present study. The lack of significant effects in the present study may also be due to a relatively low sensitivity of the zebrafish compared to rainbow trout (Elonen *et al.*, 1998; Henry *et al.*, 1997; Spitsbergen *et al.*, 1991).

Both the Baltic Sea mixture and TBDD induced AHR-regulated genes and EROD activity. A temporal increase in EROD activity has been reported for Baltic Sea perch that lives in coastal areas of the Baltic proper (Hansson *et al.*, 2006). In these areas, also high levels of PBDDs have been detected (Haglund *et al.*, 2007). However, no correlation has been established between PBDD levels and EROD activity in wild perch (Haglund *et al.*, 2010). In the present thesis, males and females responded differently regarding induction of AHR-regulated genes and EROD activity, which point to the importance of taking gender into account during experimental design and data interpretation.

Exposure to TBDD resulted in increased GR activity, which indicates induction of oxidative stress (Stephensen *et al.*, 2002). Several stress responsive genes, i.e. *mt* and *hsp70* (Gupta *et al.*, 2010; Haq *et al.*, 2003), were also upregulated in zebrafish after exposure to TBDD but not after exposure to the Baltic Sea mixture.

Exposure to TBDD reduced spawning success, altered ovarian morphology and reduced hepatic Vtg gene expression, which indicates that TBDD has a similar effect pattern as the chlorinated analogue (King Heiden *et al.*, 2006). The reduced hepatic Vtg gene expression correlated well with the reduced ovarian maturity. The decreased Vtg gene expression in TBDD-exposed fish might be due to interference with the steroidogenic pathway, which has been shown for TBDD *in vitro* (Ding *et al.*, 2007), and/or might be a result of liver toxicity, which has been induced by TBDD in rat (Ohbayashi *et al.*, 2007) and by TCDD in zebrafish (King Heiden *et al.*, 2006; Zodrow *et al.*, 2004). The present thesis did not investigate liver histopathology, but TBDD exposed females had increased hepatic expression of the *saa1* gene, which is regarded as a potential biomarker of liver toxicity (Hayes *et al.*, 2007; Jensen *et al.*, 1997).

Overall, the results show that dietary exposure of adult zebrafish to sublethal concentrations of PBDDs may impair reproductive physiology, induce AHR-regulated genes, EROD activity, and oxidative stress. Although effects were seen at concentrations higher than those measured in the Baltic Sea, the similar effect pattern as compared to the chlorinated dioxins imply that the PBDDs possibly may add to the already high PCDD concentrations and thereby cause additive effects. Moreover, due to the relatively low sensitivity of zebrafish to AHR agonists, both regarding CYP1A induction and effects on early-life stages (Jönsson *et al.*, 2009; Elonen *et al.*, 1998), complementary studies investigating effects of PBDDs on native fish species should be performed to estimate the risk of PBDDs for Baltic Sea fish.

5 Concluding remarks

5.1 Exposure routes

Hydrophobic chemicals in the aquatic environment tend to adsorb to suspended solids and sediments, and in laboratory studies to test chamber walls. For compounds with log K_{ow}>6, oral exposure is in general considered to be the most important route of uptake in fish, whereas compounds with log K_{ow} between 3 and 6 are considered to be taken up both via gills and the GI. Most of the brominated compounds studied in the present thesis are highly hydrophobic (log K_{ow} >6), such as the PBDDs and most of the BFRs, and thus, dietary exposure is likely ecologically relevant. For the BFRs in the lower log K_{ow} range (<6), such as TBP and TBECH, both water exposure and exposure via feed might be significant. In the embryo toxicity test, in which embryos were exposed via water, effects were detected for BFRs with log K_{ow} between 4 and 7, whereas the most hydrophobic compounds could not be adequately assessed. Due to the large variation in chemical properties between the different compounds in the BFR mixture feed, it cannot completely be excluded that some of the compounds may partition from feed to the water. In order to minimize such water contamination, water was changed daily, small feed lots were given, and food debris was removed regularly.

Most of the brominated chemicals exposed via feed were retained in adult zebrafish. However, the retention was generally low with a few exceptions, e.g. particularly for BDE 28, HBCD, TBDD and 1368-TeBDD, all of which either had log K_{ow} between 6 and 8, or had a molecular structure with sterical hindrance for metabolism, or both.

Maternal transfer has previously been described for hydrophobic compounds, such as PCBs, PBDDs and PCDDs. In the present thesis, most

of the brominated compounds detected in female fish were also found in their offspring, and may thereby affect the fish embryos at a critical period during development. The concentration in eggs relative to fish, varied between the brominated compounds tested. For the BFRs in the mixture, hydrophobic compounds were transferred to a higher extent than less hydrophobic compounds. The BFRs studied represent a wide range of BFRs, which suggests that other BFRs may also be transferred from fish to eggs. For the PBDDs, no relation between transfer and number and position of the bromine atoms could be found, nor could a relation between transfer and hydrophobicity. The mechanisms of maternal transfer, which were not studied here, should be considered in future studies.

In conclusion, the choice of exposure route should be based on the properties of the chemicals to be evaluated, and for PBDDs and most of the BFRs, exposure via feed is recommended.

5.2 Reproduction and early life-stage development

Reproduction and early life-stage development are vulnerable periods during the life cycle, and short exposure during such periods may considerably affect development and reproductive health. Exposure to both BFRs and PBDDs significantly changed ovarian morphology, and the histopathological alterations were in general the most sensitive reproductive endpoint. In female fish exposed to PBDDs, the expression of potential biomarker genes was investigated. For reproduction, Vtg gene expression seemed to be correlated to reduction in number of vitellogenic oocytes. Further investigations on gene responses in fish with impaired reproduction may provide insight into the mechanisms behind reproductive effects and may enable the identification of early warning biomarkers. Among the reproductive output parameters, the most sensitive was fertilization success. Spawning success was only significantly affected in TBDD exposed groups. In the BFR study, fish were exposed to high concentrations during a short period. To better estimate the risk of the BFRs on reproduction, future studies should be based on long-term exposure at lower concentrations.

Although most of the brominated compounds were maternally transferred, minor effects were in general seen on early life-stage development in offspring. The low response may be due to low concentrations of the brominated chemicals in the eggs or, for the PBDDs, a low sensitivity of zebrafish to AHR agonists. However, the reduced hatching success in offspring from adult zebrafish dietary exposed to the BFR mixture shows that maternal transfer of BFRs may affect early life-

stage development in fish. Moreover, the embryo toxicity test revealed novel effects on fish early life-stage development of waterborne TBP, TBECH, and TBBPA OHEE, and confirmed previously reported effects of TBP and TBBPA. These results suggest that fish early-life stages also may be affected by BFRs via uptake through the chorion. TBECH was maternally transferred and affected embryos via water exposure. The occurrence of TBECH in various environmental compartments, and its potential anti-androgenic effects, warrants further investigation of its biological effects.

5.3 Mixtures versus individual compound exposure

Exposure to single chemicals is often used in risk assessment but does not reflect the actual chemical exposure situation for fish in the environment, where a complex mix of substances would be expected. The composition of defined mixtures could ideally be based on mode of action or on known environmental concentrations. This thesis investigated two defined mixtures. The PBDD mixture was designed to reflect levels in biota, and contained compounds which are expected to have the same mode of action. The BFR mixture, although consisting of many environmental relevant compounds, was neither based on known environmental concentrations nor on one specific mode of action. Instead, the BFR mixture consisted of compounds with a variety of potential endocrine disrupting mode of actions, of which some BFRs have shown *in vitro* effects on both estrogen- and androgen-mediated processes. For many of the BFRs, however, *in vivo* effect concentrations are lacking. The BFRs in the mixture were added in equal molar amounts to be able to compare uptake and maternal transfer of the BFRs. In future studies, it would be beneficial to expose fish to individual BFRs to obtain compound-specific data, which in turn could be used in effect-based studies of mixture toxicity, both on adults and maternally exposed offspring.

5.4 Zebrafish as a model for prediction of reproductive health in wild fish

The use of zebrafish as a model species makes it possible to perform studies of consecutive generations during a relatively short time. The reproduction strategy in the tropical zebrafish, however, differs from most species native in Swedish waters, such as perch, pike, roach and salmonids, which generally take several years until they first spawn and then only spawn maximum once per year. Although the mode of action of EDC is conserved among

vertebrates at the molecular level, the difference in reproduction biology might imply that the consequences at the population level can differ between species, e.g. between zebrafish and salmonids. Moreover, compared to salmonids, zebrafish eggs contain relatively low quantity of lipids and, therefore, a lower quantity of lipophilic organic toxicants may reach the embryo. This, together with the relatively low sensitivity of zebrafish to AHR agonists compared to salmonids, suggests that future investigations should focus on effects of PBDDs on native species.

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