Till Agnes, Julia och Frida

Abstract

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A large number of chemicals can contaminate aquatic environments and therefore be exposed to fish and amphibians during their sensitive stages of development. This raises the need for robust methods to identify chemicals that disturb the developmental process. In this thesis, methods for toxicity testing and biomonitoring were developed for zebrafish (*Danio rerio*) and West-African clawed frog (*Xenopus tropicalis*). Using these methods, two groups of substances that have achieved attention during recent years were tested, synthetic musks and brominated flame retardants, as well as substances with known mechanism of action. Moreover, zebrafish embryos were used to evaluate chemically complex extracts prepared of effluent water from oil/gas production platforms. Exposure was performed on the embryo stages, to reveal embryo toxic endpoints and in connection to the metamorphosis process in frogs, to evaluate disturbances of the thyroid hormone system. Both methods were able to detect adverse effects in exposed animals. The studies showed that some musk substances had toxic effects on embryos in environmentally relevant concentrations. Embryo toxic responses of musk ketone (MK) and tetrabromobisphenol-A (TBBPA) were recorded in zebrafish as well as in *Xenopus tropicalis* and moor frog (*Rana arvalis*) at comparable concentrations. Zebrafish embryos were adequate for monitoring the toxic impact of effluent water from oil/gas production platforms. Effects on *X. tropicalis* tadpoles due to exposure to propylthiouracil were reduced development and decreased hind limb length, which can be explained by thyroid disruption. Increased sensitivity of the method was achieved by measurements on histological preparations of the thyroid glands. Exposure to polybrominated diphenylethers resulted in signs of thyroid disrupting properties of one tested congener, BDE-99. Moreover, distribution of BDE-99 in tadpole and juvenile *X. tropicalis* showed long-term retention and accumulation in adipose tissue.

Keywords: zebrafish, *Danio rerio*, *Xenopus tropicalis*, embryo, toxicity, tadpole, thyroid, musks, brominated flame retardants

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Appendix

Papers I-V

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

I. Carlsson, G. & Norrgren, L. (2004). Synthetic Musk Toxicity to Early Life Stages of Zebrafish (*Danio rerio*). *Archives of Environmental Contamination and Toxicology 46,* 102-105.

II. Carlsson, G. & Norrgren, L. (2007). Embryo toxicity using different classes of aquatic vertebrates. *Archives of Environmental Contamination and Toxicology,* Submitted.

III. Carlsson, G., Norrgren, L., Hylland, K. & Tollefsen, KE. (2007). Effluents from oil production platforms in the North Sea cause developmental toxicity to zebrafish (*Danio rerio*) embryos. Manuscript.

IV. Carlsson, G. & Norrgren, L. (2007). The impact of the goitrogen 6-propylthiouracil (PTU) on West-African clawed frog (*Xenopus tropicalis*) exposed during metamorphosis. *Aquatic Toxicology 82,* 55-62.

V. Carlsson, G., Kulkarni, P., Larsson, P. & Norrgren, L. (2007). Distribution of BDE-99 and effects on metamorphosis of BDE-99 and -47 after oral exposure in *Xenopus tropicalis*. *Aquatic Toxicology,* Submitted.

Abbreviations

ACTH	Adrenocorticotropin
ADBI	Celestolide
AHTN	Tonalide
ANOVA	Analysis of variance
BDE	Brominated diphenylether
BFR	Brominated flame retardant
bw	Body weight
CMC	Carboxymethylcellulose
CORT	Corticotropin
CRH	Corticotropin releasing hormone
DMSO	Dimethylsulphoxide
dpf	Days post fertilisation
FETAX	Frog embryo teratogenicity assay-Xenopus
GW	Glass wool
HCG	Human chorionic gonadotropin
HBCD	Hexabromo cyclododecane
HHCB	Galaxolide
hpf	Hours post fertilisation
LC_{50}	Lethal concentration (50% mortality)
LOEC	Lowest observed effect concentration
MK	Musk ketone
MS-222	tricaine methane sulphonate
MX	Musk xylene
PBB	Polybrominated biphenyl
PBDE	Polybrominated diphenylether
PCB	Polychlorinated biphenyl
PTU	6-Propylthiouracil
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SPE	Solid phase extraction
STW	Sewage treatment work
T3	Triiodothyronine
T4	Thyroxine
TRα	Thyroid receptor α
TRβ	Thyroid receptor β
TBBPA	Tetrabromobisphenol-A
TCDD	Tetrachlorodibensodioxine
TRH	TSH releasing hormone
TSH	Thyroid stimulating hormone
TTR	Transthyretin
XEMA	Xenopus metamorphosis assay

Introduction

General introduction

Many reports concerning adverse health effects and population declines in fish have been published as reviewed by Norrgren *et al.* (1998). It has been proposed that these problems are mainly because of chemical contamination in many cases. Examples include declines in population size (Swales *et al.,* 1998) weak reproductive success (Spies & Rice, 1988), disruption of gonad development (Jobling *et al.,* 2002) and larval mortality (Cook *et al.,* 2003). Recently, the global problem of amphibian population declines has received much attention. Different explanations have been suggested, including pathogens (Lips *et al.,* 2006), habitat degradation (Delis, Mushinsky, & McCoy, 1996), dryness associated with climate change (Rohr & Madison, 2003) and chemical contamination (Alford & Richards, 1999; Hayes *et al.,* 2003). Both fish and amphibians are animal classes that occupy aquatic ecosystems. Fish live their entire life in water, whereas amphibians in general breed and have the embryo and larval periods in water while the main adult life is terrestrial. Many anthropogenic chemicals that are released end up in aquatic environments. Fish and amphibians might thus be exposed to a number of different chemicals, originating from different sources. Reproduction including development of the offspring during the embryo and larval stages are considered to be very sensitive stages in the life cycle to chemical exposure (von Westernhagen, 1988; Nagel, 2002). Some substances have been reported to be able to disrupt endocrine systems in animals, which has lead to intense research in this field, mainly focused on disruption of sex hormones, sexual differentiation, and the reproductive system. (Vinggaard *et al.,* 2000; Örn *et al.,* 2003; Meucci & Arukwe, 2005; Andersen *et al.,* 2006). Disruption of the thyroid system has been of less concern, but in recent years several studies propose that chemicals may act on thyroid mechanisms both in vivo and in vitro. Further, other studies of unusual thyroid gland development and ratios of circulating thyroid hormones suggest a thyroid disruption in natural populations which is often correlated with high concentrations of organic chemicals (Rolland, 2000; Colborn, 2002). Several laboratory studies propose that a number of environmental agents may act on thyroid mechanisms such as perchlorate (Goleman et al. 2002; Bradford *et al.,* 2005; Crane *et al.,* 2005), polychlorinated biphenyls (PCBs) (Crisp *et al.,* 1998; Brown *et al.,* 2004), tetrachlorodibenzodioxine (TCDD), and chlorinated pesticides (Crisp *et al.,* 1998).

The huge numbers of chemicals in circulation that might end up in aquatic biota raises the need for robust methods for risk assessment of chemicals that might negatively affect different animal phyla. Further, the increasing number of reports on fish and amphibian declines on the population levels makes it important to include these animals accurately in test strategies that could be applied both for regulatory assessment and as a support in connection with environmental monitoring programs. The new chemical legislation in Europe, Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), will result in toxicology testing of thousands of previously untested substances to provide

information background for the use in risk assessment. Thus, there is a huge requirement for reliable, robust and relevant test methods and strategies for validation of chemicals. Among OECD test guidelines, a number have been developed for the use in aquatic ecotoxicity testing to assess the potential effects of chemicals. OECD Test Guidelines may also be used for other purposes, including in connection with testing of environmental samples and in fundamental research (OECD, 1998). Efforts have been made in increasing sensitivity and specificity in such tests by the implementation of additional sub-lethal endpoints reflecting hormone disruption effects, behaviour alterations, physiological effects and reproduction disorders (Darland & Dowling, 2001; Peitsaro *et al.,* 2003; Örn *et al.,* 2003; Fort *et al.,* 2004a, b; Holbech et al., 2006), which in the long run might be of equal ecological relevance as lethality, although observable at much lower concentrations.

Fish embryo test as a model for developmental toxicity

Early development tests

The acute fish test (OECD, 1992a) has for a long time been a major component in toxicity testing (Braunbeck, 2005), in which acute chemical toxicity is determined as a 96-h LC_{50} value. However, the environmental significance of death of individuals after short-term exposure to high concentrations is questionable (Nagel, 2002). Further, the use of acute toxicity involves problems of ethical concern when animals are subjected to considerable pain and suffering (Nagel, 2002). It has been suggested that we should focus on tests that show sub-lethal effects and more sensitive endpoints related to early development (Dahl *et al.,* 2006). Early developmental tests in fish might be regarded as less ethically problematic (Braunbeck, 2005) and generally more sensitive (von Westernhagen, 1988; Nagel, 2002) than tests on adult fish. Standardised guidelines for toxicity tests using embryos and larvae of both fish and amphibians have been developed (OECD, 1992b; ASTM 1999; ISO 1999). These test systems are fast to perform, relatively inexpensive, but still sensitive and ecologically relevant.

Fish embryo test

The fish embryo test is based on the idea for zebrafish (*Danio rerio*) suggested by Schulte & Nagel (1994). The basic principle is to expose eggs from fertilisation until completion of embryogenesis at 48 h post fertilisation (hpf). Exposure is performed in well plates in small volumes of media. Individual monitoring is performed at regular time intervals, where various responses are recorded (Schulte & Nagel, 1994). The endpoints can be separated in two types, lethal and sub-lethal (Nagel, 2002). Lethal endpoints include coagulation of egg, tail not detached from yolk, lack of somites and no heart beats. Further, sub-lethal endpoints that might indicate the mode of action of the toxic response can be measured such as completion of gastrulation, eye development, spontaneous movement, circulation, pigmentation, oedema and heart rate (Nagel, 2002). The possibility of observing certain action modes might help to identify relevant classes of chemicals in extract samples with unknown content. Further expansion of the test can be made by prolonging the time of experiment and including measurements such as presence

of spinal deformations (Hollert *et al.,* 2003), pericardial area (Fraysse, Mons, & Garric, 2006) and body or tail length (Nagel, 2002; Fraysse, Mons, & Garric, 2006). The fish embryo test has been standardised in Germany for the use in routine effluent monitoring. Standardisation is under consideration in ISO and OECD of the method for sewage water and chemical testing (Braunbeck *et al.,* 2005). The methodology has been used for example in toxicology test of environmentally relevant chemicals (Cook, Paradise & Lom, 2005; Fraysse, Mons, & Garric, 2006; Kapp *et al.,* 2006) and tests of sediment and sediment extracts (Hallare *et al.,* 2005; Kammann, 2004). Braunbeck et al. (2005) showed that this protocol can also be adapted for two other fish species recommended by OECD, the Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*). Data obtained from the three species were considered to be comparable (Braunbeck *et al.,* 2005). Generally, this assay shows good correction with the conventional 96 h fish test (OECD guideline 203) (OECD 1992a), but with higher sensitivity, and it has been suggested to replace the conventional test (Lange *et al.,* 1995; Braunbeck *et al.,* 2005).

Frog metamorphosis as a model to evaluate thyroid disruption

Thyroid hormone system

Thyroid endocrinology and biochemistry are very similar among vertebrates although specific molecules differ (Zoeller & Tan, 2007). Thyroid hormones are crucial for homeostasis maintenance. Altered status may affect basal and lipid metabolism and cardiovascular, gastrointestinal and muscle functions. During growth and development, thyroid hormones are important for maturation of the brain (Amaral Mendes, 2002). In general among vertebrates, the processes in the thyroid gland are regulated by secretion of thyroid stimulating hormone (TSH) from the adenohypophysis. This hormone is regulated by TSH releasing hormone (TRH) from neurosecretory cells in hypothalamus. In the thyroid gland, peroxidases convert monoiodotyrosine and diiodotyrosine to T3 and T4, which are released in the circulation (Eckert, Randall & Augustine, 1988). Some enzymes control metabolism, homeostasis and excretion of thyroid hormones, including deiodinases (Huang, Marsh-Armstrong & Brown, 1999; Campos-Barros, 2000), UDP-glucuronyl transferases and sulfotransferases (Brouwer et al, 1998). In frog tadpoles, the control of release of TSH is by corticotropin releasing hormone (CRH), which stimulates both TSH and adrenocorticotropin (ACTH). Further, corticosterone (CORT) which is produced in the adrenal gland as response to ACTH, can cause negative feedback on the pituitary and decrease both TSH and ACTH (Hayes, 2000). This is not observed in adult amphibians, where TSH is controlled by TRH and ACTH is controlled by CRH, separately. Among vertebrates, thyroid hormones bind to plasma proteins in serum such as thyroxine-binding-globulin, transthyretin (TTR) and albumin, which promotes transport to target tissues (Capen, 1996; Meerts *et al.,* 2000). TTR have been recorded as the major thyroid hormone binding protein in plasma of metamorphosing tadpoles (Yamauchi *et al.,* 1993) and in some fish species (Yamauchi *et al.,* 1999). Two genes, TRα and TRβ, code for the nuclear thyroid receptor that mediate thyroid

hormone action, which can be up-regulated as a response to increasing levels of thyroid hormones (Yaoita & Brown 1990).

Metamorphosis

The metamorphosis in amphibians is controlled by thyroid hormone (Wong & Shi, 1995; Hayes, 2000). This gives unique opportunities to assess apical characteristics during the metamorphosis process, to reveal disruptions of the thyroid hormone system. The metamorphosis process in Xenopus laevis begins around stage 54 (Nieuwkoop & Faber, 1994), shortly after formation of the thyroid gland, when endogenous thyroid hormones are detectable (Leloup & Buscaglia, 1977). However, tadpoles can respond to exogenous thyroid hormones at earlier stages (Shi *et al.,* 1996), which suggests that experimental chemical exposure should include the period before and after this developmental stage, to optimise the possibility of detecting both thyroid hormone agonists and antagonists. Xenopus laevis has been the major amphibian species for detection of thyroid disrupting chemicals (Opitz *et al.,* 2005; Degitz *et al.,* 2005; Goleman *et al.,* 2002; Goleman, Carr & Anderson, 2002; Tietge *et al.,* 2005). A main endpoint in these studies has been the developmental stage. In addition, apical endpoints such as hind limb length, body length, biochemical measurements reflecting concentrations of thyroid hormones (T3 and T4) (Opitz *et al.,* 2006) and histopathological evaluation of thyroid gland (Degitz *et al.,* 2005; Tietge *et al.,* 2005; Opitz *et al.,* 2006) have been applied. Different test approaches, including tail resorption assay, tail tip regression and Xenopus metamorphosis assay (XEMA), have been reported in Xenopus tests of this kind.

Tail resorption assay

Xenopus laevis tadpoles were exposed at metamorphic climax starting from stage 58 for 14 days (Fort *et al.,* 2000). Video images captured the tadpoles during the test and tail length was measured at stage 63, 64, 65 and 66. Median inhibitory concentration (IC_{50}) and median stimulatory concentration (SC_{50}) were determined. This test proved to be powerful in detecting several substances that were both stimulatory and inhibitory. Further, the use of additive T3 resulted in detecting whether inhibitions were reversible or not (Fort *et al.,* 2000). A refinement of this method protocol has been reported (Balch *et al.,* 2006) where morphometric data were evaluated as cumulative resorption ratio (CRR) and proportion of metamorphosed frogs (PMF). Tadpoles were exposed beginning already at stage 50 although the actual monitoring of CCR was performed between stage 58 and 66.

Tail tip regression

Tadpoles were arrested in developmental stages 53-54 by the use of methimazole. Tail tips were cut off and were left in 24 well plates with culture media for 24 h before exposure. Exposure of the tested chemicals was performed in well plates with or without addition of T3 during 7 days. Tail tips were measured every day using a millimeter grid. Main endpoint was regression of tail tip (Schriks, 2006).

Xenopus metamorphosis assay (XEMA)

In the initial report of the XEMA test, Xenopus laevis tadpoles were exposed starting at stages 48 to 50 for 28 d. Tadpoles were raised in 10-L aquaria in groups of 30 individuals. Developmental stage and whole body length at 7, 14, 21 and 28 days of exposure were the main endpoints (Opitz *et al.,* 2005). Another approach was used by Degitz et al. (2005) where tadpoles were exposed from either stage 51 or 54 for 14 or 21 days. Developmental stage, body weight and histological evaluation of thyroid glands were parameters measured. Opitz et al. (2006) measured time to fore limb emergence as endpoint and continued exposure until each animal reached stage 58. Mitsui et al. (2006) and Jagnytsch et al. (2006) used hind limb length as measurement of developmental progress.

The animals

Zebrafish (Danio rerio)

Zebrafish is a small cyprinide from south-east Asia. It measures 3-5 cm and is insensitive to change in water quality and easily obtainable to a low cost. Zebrafish is easily maintained in laboratories with simple equipment; it breeds all year round and eggs can be collected in large quantities. The life cycle is rapid under optimal rearing conditions. These advantages have led to the widespread use of this species in toxicology testing, including standardised protocols (Braunbeck *et al.,* 1990; Carlsson *et al.,* 2000; Örn *et al.,* 1998; Örn *et al.,* 2003; ISO, 1999; Diekmann *et al.,* 2004). Embryos of zebrafish are transparent, which makes it possible to monitor individual organs during development using a standard stereo microscope.

West African clawed frog (Xenopus tropicalis)

The South African clawed frog (*Xenopus laevis*) has been widely used as an amphibian laboratory animal in different disciplines, including standardised protocols such as Frog Embryo Teratogenicity Assay-*Xenopus* (FETAX) (ASTM, 1999). During recent years, another *Xenopus* species, the West African clawed frog (*X. tropicalis*) has been introduced mainly for genetic studies because of its diploid genome, in contrast to the tetraploid *X. laevis* (Amaya, Offield & Grainger, 1998; Fort *et al.,* 2004c). Further, the short period from fertilisation to sexual maturation makes *X. tropicalis* a good candidate for life-cycle studies (Fort et al. 2004b). Comparisons have been made between the two *Xenopus* species indicating similar response and sensitivity regarding early development toxicity (Song *et al.,* 2003; Fort *et al.,* 2004c) as well as in partial life-cycle studies including sexual maturation (Pettersson et al. 2006). The defined normal developmental process for *X. laevis* (Nieuwkoop & Faber, 1994) has been applied and appears to be appropriate also for *X. tropicalis* (Fort *et al.,* 2004b; 2004c; Mitsui *et al.,* 2006). Some studies on thyroid effects using *X. tropicalis* (Fort *et al.,* 2004b; Mitsui *et al.,* 2006) indicate comparable responses as *for X. laevis*. *X. tropicalis* is easy to keep under laboratory conditions. It can be induced to breed by injections of human chorionic gonadotropin (HCG) all year round and produces large quantities of eggs.

Risk chemicals

Aquatic environments are influenced by human activities and modern life style, which have resulted in extensive use of chemicals, not only in industry but also in household products. Synthetic musk compounds and brominated flame retardants (BFRs) are examples of chemicals that have been detected in environmental samples at high concentrations (Gatermann *et al.,* 1999; deWit, 2002).

Musks

Musks are used as perfuming agents in consumer products, such as cosmetics, toiletries, detergents, and soaps. They are divided into nitro musks, polycyclic musks, and macrocyclic musks, all of which are chemically unrelated but have the same typical musk odor. The nitro musks, including musk xylene (MX) and musk ketone (MK) have been recorded in water (Yamagishi et al. 1981; Paxéus, 1996; Heberer, Gramer & Stan, 1999), suspended particulate matter (Winkler *et al.,* 1998), water-living organisms (Yamagishi et al. 1981; Franke *et al.,* 1999; Gatermann *et al.,* 1999), human blood (Angerer & Kaefferlein, 1997), and human breast milk (Rimkus & Wolf, 1996). In recent years, some polycyclic musks, i.e., tonalide (AHTN), galaxolide (HHCB) and celestolide (ADBI), have also been detected in environmental samples (Hajslova *et al.,* 1998; Heberer, Gramer & Stan„ 1999; Fromme *et al.,* 2001). The chemical characteristics indicate that these substances are persistent and have the potential to bioaccumulate. The highest concentrations have been detected in effluents from sewage treatment works (STWs), indicating that this is the major entrance for these substances to the aquatic environment. Recordings of HHCB and AHTN in water samples from or in connection with STW effluents in Berlin showed concentrations around 1 µg/L (Fromme *et al.,* 2001). Further, in a study of waters in Berlin, HHCB, AHTN, and ADBI were detected in all samples and MK in most (Heberer, Gramer & Stan, 1999). Concentrations of HHCB and MK in water outside three Swedish STWs have been recorded to 1-6 µg/L and 1-5 µg/L, respectively (Paxéus, 1996). A recent study indicates that the concentrations of MK and MX are declining (Ricking *et al.,* 2003). However, the occurrence of musks in water and the high bioconcentration potential of these substances raise concern about possible effects on aquatic organisms.

Brominated flame retardants (BFRs)

BFRs are used as additive flame retardants in a wide variety of commercial and household products such as plastics, textiles, and electronic appliances including computers and televisions (Minh *et al.,* 2007). Studies have shown high concentrations of BFRs in the environment and this indicates a potential health risk to organisms exposed to these environmental contaminants (de Wit, 2002; McDonald, 2002). The BFRs identified as of particular environmental relevance are polybrominated biphenyls (PBBs), hexabromocyclododecane (HBCD), tetrabromobisphenol-A (TBBPA) and polybrominated diphenyl ethers (PBDEs) (de Wit, 2002). Total concentrations of PBDEs have increased exponentially in the past years in the aquatic biota with predominance of tetra and penta congeners (Ikonomou, Rayne & Addison, 2002). Contamination by PBDEs can now be

regarded as ubiquitous. These can be found around the world in air, water, fish, birds, marine mammals, and humans (Hites, 2004). It has been suggested that these chemicals bioconcentrate in lipid-rich tissues (Haglund *et al.,* 1997). The extensive use and the increased levels in the environment of these chemicals have raised concerns about these chemicals as environmental contaminants. TBBPA has been detected in aquatic organisms (Morris *et al.,* 2004), sediment and sewage sludge (Sellström & Jansson, 1995) but are often below limit of detection in natural water (Kuiper *et al.,* 2007) which suggests exposure through other sources. Kuiper et al. (2007) recorded negative effects in reproduction parameters, including offspring survival, on adult zebrafish with environmentally relevant body burdens of TBBPA (5-7 µg/g lipid). Hydroxylated metabolites of PBDEs as well as TBBPA have been found to be potent competitive inhibitors of binding to TTR similar to certain PCBs (Lans *et al.,* 1993; Meerts *et al.,* 2000; Hakk, Larsen & Klasson-Wehler, 2002). TBBPA has also been shown to inhibit binding of triiodothyronine (T3) to mammalian thyroid receptor (Kitamura *et al.,* 2005) Moreover, PBDEs caused decreased serum thyroxine (T4) and T3 levels as well as decreased colloid area and increased follicular cell heights in the thyroid glands in pubertal rats (Stoker *et al.,* 2004).

Aim

The overall aim of the present thesis was to develop and refine test models based on exposure during early life stages in fish and amphibians which could be applied for evaluation of chemicals and environmental samples.

Materials and methods

Chemicals

4-*tert*-butyl-2,6-dimethyl-3,5-dinitrophenylethanone (musk ketone, MK) and 6-propylthiouracil (PTU) were purchased from Sigma Aldrich Sweden AB. 1-*tert*-butyl-3,5-dimethyl-2,4,6-trinitrobenzene (musk xylene, MX) and 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-[g]-2-benzopyrane (Galaxolide, HHCB) were bought from Promochem AB, Kungsbacka, Sweden. 1-(5,6,7,8-tetrahydro-3,5,5,6,8,8-hexamethyl-2-naphthalenyl)ethanone (Tonalide, AHTN) were provided from Huber the Nose, Zumikon, Switzerland. Tetrabromobisphenol-A (TBBPA); 2, 2', 4, 4'-tetrabromodiphenyl ether (BDE-47); 2, 2', 4, 4', 5-pentabromodiphenyl ether (BDE-99) and radiolabelled ^{14}C-BDE-99 (specific radioactivity 49 Ci/mol) were kindly given by Prof. Åke Bergman, Department of Environmental Chemistry, Stockholm University, Sweden. Stock solutions of MK, MX, AHTN, HHCB and TBBPA were prepared in dimethylsulphoxide (DMSO). PTU was dissolved in water. BDE-47, BDE-99 and ^{14}C-BDE-99 were first dissolved in ethanol. These polybrominated diphenylethers (PBDEs) were mixed with feed in appropriate concentrations whereupon ethanol was evaporated. Produced waters were collected from nine oil platforms and one gas and condensate production facility in the North Sea at two separate occasions 6 weeks apart (sample series 1 and 2). Extracts of glass wool filtered effluent including oil and particulate matter (GW extracts) and water soluble fractions of effluent (SPE extracts) were prepared by NIVA, Oslo Laboratory, Norway, according to Thomas et al. (2001). Stock solutions of extracts were prepared in DMSO.

Adult animals

All use of animals was performed at the Department of Biomedical Sciences and Veterinary Public Health, SLU. Adult zebrafish (*Danio rerio*) (Paper I-III) was acquired from a local supplier in Uppsala, Sweden, and maintained in aquaria with temperated water (26±1°C) and in a 12-h light/dark cycle. Standardised water according to ISO (1996) was used for adult zebrafish and for embryos/larvae in all experimental studies. Adult fish were fed freeze-dried chironomids (Nutrafin®) and commercial flake feed (Sera®) on a daily basis. Adult West African clawed frog (*Xenopus tropicalis*) (Paper II, IV and V) was purchased from Carolina Biological Supply Company, Burlington, North Carolina, USA. They were maintained in aquaria with a flow-through system of carbon-filtered tapwater. Water temperature was (26±1°C) and the light/dark cycle was 12 h. Adult *X. tropicalis* were fed three times a week with salmon granulates (Aller 514, Aller Aqua, Sweden).

Breeding, eggs and tadpoles

Adult zebrafish of both sexes were selected and transferred to cone-shaped breeding funnels with 20-25 L of standardised water, on the day before breeding. Each breeding group included 5-10 females and 10-15 males. Each funnel was

provided with a net, separating adult fish from their eggs. After mating, which started at onset of light, the eggs fell to the bottom of the reproduction funnel. Between 30 and 60 min after light was turned on, all eggs present in the reproduction funnel were collected by opening a bottom tap. *X. tropicalis* was induced to breed using human chorionic gonadotropin (HCG). Two days prior to mating, 20 IU of HCG was administered into the dorsal lymph sac to adult males and females. This was followed by a dose of 100 IU immediately before introducing the frogs to breeding aquaria. After breeding, parents were removed and eggs were collected (Paper II) or left to develop until they hatched (Paper IV and V). For raising tadpoles, individuals were collected at 2 days post fertilisation (dpf) and raised together in groups of 50 in aquariua containing 10 L tadpole water. Tadpole water was prepared by 25% carbon-filtered tapwater mixed with 75% deionised water. Tadpoles were fed approximately 4% of their body weight (bw) per day with a water suspension of Sera micron (SERA®) starting 2 dpf. After metamorphosis, juvenile frogs were fed with salmon granulates. Moor frog (*Rana arvalis*) eggs (Paper II) were collected from a natural pond in spring. Eggs were taken from different clutches and kept separate one week in a refrigerator (+4°C).

Larvae hatching and survival test

Zebrafish eggs were exposed to the tested chemical in petri dishes, each initially with 30 eggs. After 1 dpf unfertilised eggs were removed and the number was reduced to a maximum of 20. Numbers of hatched/unhatched and dead/alive individuals were recorded at least daily for each petri dish. Larvae were not fed during the study, so all individuals eventually died. Primary endpoints were hatching time and survival time. MK, MX and AHTN were tested in concentrations ranging in between 1-100, 0.1-33 and 1-100 µg/L, respectively (Paper I).

Embryo tests

Zebrafish eggs were immediately exposed to the tested substance/extract in solvent and water. The eggs were examined in a stereo microscope for selection of eggs that had reached at least the four-cell stage. Selected eggs were placed individually in 96-well plates, one egg in each U-formed well, with 250 µL solution. The eggs were examined in a stereo microscope at 24 and 48 hpf. Microscopic examination was performed by use of a light source equipped with a fiber optic cable to prevent temperature stress of the embryos. Studied endpoints at different observation times are summarised in Table 1. After these observations, a camera recorded the approximate hatching time of each embryo by photographing at 1 to 2 hours intervals (Paper II and III). At seven dpf, additional monitoring was performed and the test was terminated (Paper III).

Collected *X. tropicalis* eggs (Paper II) were treated in L-cystein (Sigma-Aldrich Sweden; 1.25 g in 50 mL water) to remove the jelly coat. Water used for *X. tropicalis* embryos were FETAX water (ASTM, 1999) diluted 1:1 with deionised water. Eggs considered to be fertilised by observation in stereo microscope were

selected and distributed to beakers with the test substances dissolved in water. Embryos were transferred individually to wells in 96-well plates together with 250 µL solution. Observations corresponding to the zebrafish test were performed at 24 and 48 hpf and time-lap photographing for hatching time monitoring were performed between 10–46 hpf (Table 1). Termination was after observations at 48 hpf.

Moor frog eggs were exposed individually in beakers with 50 mL FETAX water including the tested chemical. Observations of mortality, hatching and heart rate were performed in stereo microscope regularly until termination after 7 days (Table 1).

Table 1. *Time after onset of exposure for observations of studied endpoints in embryo tests using zebrafish, Xenopus tropicalis and moor frog.*

Endpoints	Zebrafish	*Xenopus*	Moor frog
Mortality	24, 48 h	24, 48 h	48, 96 h, 7 d
Spontaneous movement	24 h	24 h	-
Eye development	24 h	24 h	-
Tail extension	24 h	24 h	-
Circulation	48 h	48 h	-
Pigmentation	48 h	48 h	-
Heart rate	48 h	48 h	96 h
Hatching time	48-120 h	10-46 h	24-96 h

MK, MX, AHTN and HHCB were tested on zebrafish in concentrations ranging between 1 to 1000 µg/L (Paper I). As a comparison between species, TBBPA was tested on zebrafish, *X. tropicalis* and moor frog. MK was tested on *X. tropicalis* and moor frog. Concentrations were 1 to 1000 µg/L (Paper II). Further, extracts of oil production effluents were tested on zebrafish (Paper III). Sample series 1 and 2 was tested separately. In tests of sample series 1, concentrations corresponding to about 30 times of effluent water were tested for both types of extract. For SPE extracts, a test of 3 times the effluent was tested as well. In tests of sample series 2, three concentrations were used corresponding to about 0.3, 3 and 30 times the actual effluent water for both GW and SPE extracts.

Metamorphosis tests

Stage-51 tadpoles were selected and randomly distributed in groups of 20 to aquaria containing concentrations in between 2 and 75 mg/L of PTU (Paper IV) or were kept individually in plastic jars with 400 ml tadpole water (Paper V) and exposed to PBDEs via feed. Feed concentrations were between 0.1 and 10 mg PBDE/g feed. Water renewals were made three times a week and exposure was continuing for 14 days. After 5 days of exposure, a subsampling was performed (Paper IV) and when the study was terminated the remaining individuals were sampled. All individuals were euthanized in tricaine methane sulphonate (MS 222; Apoteket AB, Sweden) Tadpoles exposed to ^{14}C-BDE-99 were treated for scintillation counting (Paper V). Sampling of other individuals involved determination of body weight and developmental stage according to Nieuwkoop and Faber (1994) (Paper IV and V). In addition, tadpoles were photographed using

a digital camera and measurements of hind limb length, total body length and snout-to-vent length were made on the photographs using Adobe Photoshop 7.0 (Adobe Systems Inc.). Eventually, tadpoles were fixed in 4% phosphate buffered formalin for histology preparations of thyroid glands.

Distribution of ^{14}C-BDE-99

Frogs were exposed at stage 54 and in stage 66 in two separate experiments. All animals were kept individually in plastic jars containing 400 mL water during the experiments. The tadpoles were exposed by adding feed mixed with ^{14}C-BDE-99, suspended in water, to the water in each jar. Exposure continued for 12 h. For exposure at stage 66, one granulate adsorbed with the chemical was dropped in each aquarium. Exposure was calculated to be 0.3 μCi (612 nmol)/g bw for each individual. After exposure period, the water in the aquaria was changed.

In both experiments individuals were sampled at 6 h, 12 h, 1 d, 2 d, 4 d, 8 d, 16 d, 32 d and 64 d after exposure. Seven individuals were analysed at each survival time except at day 64 when five individuals were sampled. Individuals were euthanized in MS 222 and wet weights were recorded. Different organs and tissues were dissected, weighed and treated for scintillation counting. Two individuals from each sampling time were embedded in carboxymethylcellulose (CMC) for whole-body autoradiography. At 64 days survival time, only sampling for scintillation counting was performed.

Histological evaluation of thyroid glands

Fixed individuals were processed for histological studies of the thyroid gland initiated by dehydration in graded series of ethanol. The specimens were embedded in paraffin blocks and transversal serial sections (5 μm) were cut through the entire thyroid gland. Sections were stained with haematoxylin-eosin and photographs in different magnification were taken using a microscope equipped with a digital camera. The maximum thyroid area for each individual was measured by measuring the area from each section from the largest region of the thyroid gland on the photographs using Adobe Photoshop 7.0. Epithelial cell height was measured in five sections approximately 10, 25, 50, 75 and 90 percent through the thyroid gland. On each section, in each of three follicles, four cells on the opposite ends of two diameters, intersecting an angle of 90º were measured. Determination of maximum follicular lumen area (Paper IV) was made by measuring the lumen areas of the largest follicle in each section starting with the section with the largest thyroid area, continuing with the five serial sections before and after leading to a mean value based on measurements of 11 section photographs.

Autoradiography

Tape section whole-body autoradiography was carried out as described by Ullberg, Larsson, & Tjälve (1982). Frontal sections were taken from ventral to dorsal side of the two individuals embedded in each CMC block. To study the distribution of non extractable radioactivity every other freeze-dried tissue section

was extracted successively with ethanol 99.5% (2 min), heptane (0.5 min, twice), ethanol 99.5% (1 min), ethanol 50% (1 min) and tap water (10 min). The sections were dried and exposed to X-ray film (Structurix; Agfa-Gevaert N.V., Belgium) together with adjacent non-extracted sections for approximately 2 months. Sections were handled at –20°C during the entire procedure until development, except for the extracted sections.

Scintillation counting

Tissues for scintillation counting were digested in 1-2 mL of Soluene-350 (Chemical instruments AB, Sweden) at 45°C for 24-72 h. Thereafter, 10 ml liquid scintillation cocktail (Hionic-Fluor, Chemical instruments AB, Sweden) was added. The radioactivity was determined by liquid scintillation spectroscopy in a liquid scintillation counter (Tri-Carb 1900CA, Packard Instrument Company, USA). Colored samples were treated with 0.3-0.5 mL of 35% hydrogen peroxide or bleaching.

Statistics

Categorical data were analysed using Fisher's exact test where response values from each treatment group were compared with the control group using Bonferroni adjustment of the p-values for compensating for multiple comparisons to the control. Continuous data were in case of normal distribution and equal variances analysed by one-way analysis of variance (ANOVA) with Dunnet's post-hoc test, comparing exposed groups with the control. Transformation of the data was performed when necessary to meet the ANOVA requirements. Ordinal data or continuous data that did not meet the ANOVA assumptions were tested by the non parametric Kruskal-Wallis test followed by Dunn's test or Mann-Whitney U-tests with Bonferroni adjustment. Tests between two groups were performed with t-test or Mann-Whitney U-test. Significance level was set to 0.05. Data were analysed using Statview 5.0.1 (SAS Institute Inc.) and MINITAB release 14 (Minitab Inc.).

Results

Larvae hatching and survival test (Paper I)

For zebrafish embryos and larvae exposed to different concentrations of synthetic musks, no effects were recorded in hatching time. Survival times were reduced in zebrafish exposed to the highest tested concentrations of MX and MK, which were 33 and 100 µg/L respectively. No effects on survival time were recorded in zebrafish exposed to AHTN up to 100 µg/L.

Embryo tests (Paper I-III)

No effects were recorded in zebrafish embryos exposed to HHCB up to 1000 µg/L. In embryos exposed to AHTN, decrease in heart rate was recorded after exposure to concentrations of 33 µg/L and higher. Decrease in heart rate was recorded in embryos exposed to MK in 10 µg/L and higher concentrations. At 330 µg/L, spontaneous movements and tail extension was reduced and in the highest tested concentration, 1000 µg/L, oedemas, absent circulation and coagulation were recorded in embryos. Embryos exposed to MX showed decreased heart rate at concentrations from 33 µg/L and higher. Further, coagulation, oedemas, absent circulation and no spontaneous movement were recorded in embryos at the highest tested concentration, 1000 µg/L (Paper I).

At 48 hpf, increased mortality was recorded in *X. tropicalis* embryos exposed to 1000 µg MK/L. Lower heart rates were recorded in groups exposed to 100 and 1000 µg MK/L. For the moor frogs exposed to MK, a decline in heart rate was recorded for individuals exposed to 10 µg/L and higher concentrations. An increased number of zebrafish embryos exposed to 1000 µg/L TBBPA showed no spontaneous movement at 24 hpf. All individuals in this group were affected in some way at 48 hpf. Oedema was present in tail and thorax in most embryos and no individual had circulation. Further, heart rate was reduced and all individuals died before hatching. *X. tropicalis* embryos exposed to 1000 µg/L TBBPA showed increased proportions of coagulated embryos, lack of circulation often including oedemas and reduced heart rate. Increased mortality was recorded in moor frogs exposed to 1000 µg/L of TBBPA, at 7 days of exposure, but no effects were found in other measured endpoints (Paper II).

In tests of sample series 1 of the extracts from production facilities in the North Sea on zebrafish embryos, GW extracts from all platforms resulted in effects on various responses. Three extracts caused increased mortality. Most extracts resulted in decreased pigmentation in embryos at 48 hpf. Decreased movement and heart rate as well as delayed hatching time were other sub-lethal endpoints where effects were recorded for embryos exposed to some extracts. All embryos exposed to the highest concentration of SPE extracts were dead at 24 hpf. In the test of 3 times the effluent concentration, embryos were unaffected according to observed endpoints in extracts from two platforms. Decreased pigmentation was observed in six treatment groups and decreased heart rates were recorded in two

groups. Mortality was increased at 7 dpf in embryos exposed to extract from the gas/condensate platform.

Five of the GW extracts from sample series 2 did not affect embryos in any of the measured endpoints even at the highest concentration tested. GW extracts from two platforms caused increased mortality in the highest tested concentration. In embryos exposed to highest concentration of GW extracts from three other platforms hatching time were delayed, in one of them decreased heart rate and reduced pigmentation were also observed. Pigmentation was reduced in embryos exposed to the median concentration of GW extracts from another platform. SPE extracts from sample series 2 resulted in increased embryo mortality in the highest concentration tested from all platforms. Exposure to SPE extracts from three platforms did not result in negative effects at lower concentrations. Increased mortality was observed in embryos exposed to the medium concentration of SPE extract from the gas/condensate platform. Sub-lethal effects were recorded in embryos exposed to medium concentrations of the rest of the SPE extracts, where pigmentation, heart rate and hatching time were negatively affected. No effects were recorded in the lowest concentration of the SPE or GW extracts.

Metamorphosis tests (Paper IV-V)

Results from the apical measurements after 5 days of exposure to PTU revealed a decrease in body length and body weight in *X. tropicalis* tadpoles in the highest tested concentration of PTU, 75 mg/L as well as a decrease in body length in tadpoles from the 5 mg/L treatment. The developmental stage was lower and the hind limb length was reduced in tadpoles sampled after 14 days of exposure to 75 mg PTU/L as compared with the control group. For other apical measurements, no effects were recorded at this sampling time. Tadpoles from all treatments (2 – 75 mg/L) had significantly larger maximum thyroid area than the control as recorded from the histology sections. Thyroid follicular cell height was significantly increased in tadpoles exposed to PTU in 5 mg/L and at higher concentrations. Increased follicular areas were recorded in 5, 10 and 20 mg/L treatments.

X. tropicalis tadpoles dietary exposed to BDE-99 or -47 showed increased mortality in the highest tested concentrations, 10 mg/g feed. Decreased developmental stage and reduced hind limb length were recorded in tadpoles exposed to 1 mg BDE-99/g feed, but no effects in body length or weight were recorded. Exposure to BDE-47 at 1 mg/g feed caused decreases in body length, body weight and hind limb length, but no effect of developmental stage was recorded. Histological examination of thyroid gland showed no effects for tadpoles exposed to 1 mg/g feed of any of the PBDE congeners.

Distribution of ^{14}C-BDE-99 (Paper V)

From both autoradiograms and scintillation measurements in frogs exposed as stage-54 tadpoles, high levels of radioactivity were recorded in liver, gall bladder, and eye melanin at initial survival times. In autoradiograms from 4 to 16 days survival times, labelling in gall bladder, kidneys and liver were decreased but

levels in melanin remained high. Further, high levels were seen in adipose tissue. At 32 days survival times, marked radioactivity was only visible in adipose tissue. Scintillation measurements of individual tissues showed corresponding results. Autoradiograms and scintillation counting from frogs exposed as juveniles showed very high concentrations of ^{14}C-BDE-99 in adipose tissues throughout the whole sampling period. Further, high concentrations were seen in the bone marrow and in the melanin of eye capsule in autoradiograms of frogs from most sampling times. Labelling was also considerable in liver, kidney and gall bladder at initial survival times, but declined later in the study. Intestines showed high labelling at early survival times both in frogs exposed as tadpoles and juveniles, but this was lower after a few days. Scintillation measurements of tissues showed a similar picture. Whole-body autoradiograms of frogs exposed both as tadpoles and juveniles and killed at all survival intervals, with tissues sections extracted with organic solvents, showed no detectable labelling.

The highest levels of total radioactivity in the whole animals were observed at 1 day and 12 h for exposures at stage 54 and 66, respectively. For tadpoles, the levels were clearly reduced from day 2 to day 64 after exposure whereas for juveniles it was no significant decline in the levels of radioactivity after 12 h. The accumulated dose of 14C-BDE-99 in the individuals at different survival times after exposures at stage 54 and stage 66 revealed that levels of radioactivity were significantly higher in the frogs exposed at stage 66 than in the tadpoles exposed at stage 54.

Discussion

Individual exposure

Individual exposure has been used in most of the studies reported in this thesis. Zebrafish and *Xenopus tropicalis* embryos have been exposed in well plates containing very small volumes of media. Moor frog eggs were exposed in larger beakers due to larger size of eggs, and both tadpole and juvenile *X. tropicalis* were exposed in 400 mL of water in plastic jars (Paper V), which corresponds to a density of 25 individuals per 10 L. In many reported studies or standardised tests using aquatic species, a number of individuals have been exposed within the same exposure unit e. g. aquarium (OECD, 1992a,b; ISO, 1999; ASTM, 1999; Opitz *et al.,* 2006; Jagnytsch *et al.,* 2006). There are several ways to treat data from such tests. Data from each aquarium can be treated as proportions (e. g. sex ratios), or as mean or median values based on measurements on all including animals, or the data analysis can be performed using a nested ANOVA, with more consideration of the response from each individual. In this approach, each aquarium is considered as one true replicate, which implies that a number of aquaria are needed within each exposure group for satisfactory data analysis. Another approach is to consider each individual as one replicate, after a test between aquaria within the same exposure group, showing no significance. This approach may be considered as pseudoreplication since several replicate values are derived from the same exposure unit (Hurlbert, 1984), where the individuals have interacted with each other. The use of one individual per exposure unit is one way of keeping the number of animals used in tests low. In the study of PTU on *X. tropicalis* (Paper IV), each replicate were initially containing 20 tadpoles. Five concentrations of PTU plus controls resulted in the use of 480 tadpoles even though each treatment only had four replicates. One advantage of this is that the use of mean values from many individuals might reduce the variation between replicates, thereby increasing the power of the statistical analysis. However, to keep the number of replicates high requires many individuals and therefore considerable work. The study on metamorphosis effects of PBDEs (Paper V), where tadpoles were exposed individually was initiated with only 60 individuals divided in six treatments and a control group, even though it resulted in eight true replicates (12 in control group) per treatment. Advantages using this strategy include reduction of the number of individuals, smaller quantities of chemicals, possibly increased statistical power due to high number of replicates, and reduction of interactions between individuals. Disadvantages are sensitivity for mortality and possible higher variation between replicates. It is not possible to compare the statistical power of the two strategies, but in the present study, negative effects were recorded in the 1 mg/g feed treatment of both BDE-47 and -99 with the use of eight replicates. The strategy of individual exposure seems to work using *X. tropicalis* at younger stages. The development rate in the metamorphosis tests in the present study corresponds to the rate in the study on PTU, where tadpoles were exposed in groups (Paper IV). However, individual exposure might not be applicable when using other species with different social or behavioural preferences. For embryos, especially of such species as zebrafish and

Xenopus, the use of individual rearing is very promising. Zebrafish embryos are possible to keep at least in as low volumes as 100 µg/L (Braunbeck, 2005), which promotes the use of exposure in low-volume well plates. The use of well plates makes it possible to keep a large number of true replicates on a small area.

Embryo tests

Assessment of endpoints

Some modifications in methodology were performed in Paper III, regarding the estimation of spontaneous movement and pigmentation compared with Paper I and II as well as other studies of this kind, where pigmentation has been estimated on a categorical level (Schulte and Nagel, 1994; Lange *et al.,* 1995). In Paper III, pigmentation was instead ranked according to an ordinal scale between 1 and 4 providing more nuances in the data. This approach involves judgements that might be subjective and preferably, the same observer should monitor this endpoint in all including tests. However, each scale level was defined as precisely as possible and this way of describing the pigmentation seems to be useful. In the tests of the extracts from production platform effluent, spontaneous movement was determined by observation of each embryo during 1 min and counting each movement, similar to the method described in Fraysse, Mons & Garric (2006). This was a problem-free approach in this study because one "movement" was very distinct in all exposure groups. This approach provided more information about the mobility of each embryo than the previously used categorical determination (Paper I & II) where the recording of movement only was determined as "yes" or "no". However, this approach might not work in general when performing this embryo method. There might be toxicants causing different kinds of movements, for example continuous shaking which may cause problems in counting or defining a "movement". Overall, there might be simple ways of replacing more of the categorical endpoints described in this method (Schulte and Nagel, 1994) into continuous or ordinal data measurements likely providing subtler information of the results obtained.

Positive control

Two main effects in several of the extracts from the first sample series tested (Paper III) were reduced pigmentation and decreased heart rate. Therefore, a positive control mixture with substances known to affect these endpoints was used in the more extensive tests of extracts from the second sample series, providing assurance of reliability of each test. In Paper I, the heart rates of zebrafish embryos exposed to musk ketone were decreased when exposed to 10 µg/L and higher concentrations. No impact on other monitored endpoints in concentrations up to, and including 330 µg/L was recorded. Further, exposure to phenylthiourea has been reported to cause reduced pigmentation in zebrafish embryos (Karlsson, von Hofsten & Olsson, 2001). Therefore a mixture of MK (50 µg/L) and phenylthiourea (7.6 mg/L) was used in the study, providing the desired results in all three tests, with clearly reduced pigmentation and reduction in heart rate. No other effects were observed in embryos exposed to this positive control mixture except decreased spontaneous movement at 24 hpf, which was observed in two of

the three tests. The responses from this positive control mixture on pigmentation and heart rate showed high repeatability in the three tests. All individuals were determined to have the highest score for lack of pigmentation in all tests and the reduction in heart rate was 78, 80 and 82% of the heart rates in the respective control groups.

Different endpoints

Hatching time for zebrafish embryos was found to be a sensitive endpoint when evaluating the complex chemical mixtures (Paper III). For the extracts tested from sample series 2, this was the most sensitive endpoint in six of the extracts, although in two cases as sensitive as other endpoints. These extracts resulted in delayed time to hatch, but using this method, a shortened hatching time is possible to detect as well. In tests of MK and TBBPA, (Paper II), no effects on the hatching time were recorded. Both delayed and premature hatching have been reported in Baltic herring (*Clupea harengus membras*) when exposed to different oil types (Linden, 1978). Prolonged time to hatch has also been reported in fish exposed to other industrial effluents (Zha & Wang, 2006) as well as after exposure to single compounds (Todd & van Leeuwen, 2002) or indirectly by maternal exposure (Nakayama *et al.,* 2005). Metals have been shown to affect hatching time in fish. Copper has been shown to cause delayed hatching in fathead minnow (Scudder *et al.,* 1988). In zebrafish, the same response has been obtained by exposure to both copper and nickel (Dave and Xiu, 1991) and to cadmium (Jörgenson, 2005; Fraysse, Mons, & Garric, 2006). However, the impact of metals on hatching time can be neglected in Paper III, due to absence of metals in the tested extracts. There have been studies on fish embryo exposure to oktylphenol and nonylphenol in concentrations up to 1000 and 500 µg/L, respectively (Gray and Metcalfe, 1999; Jörgenson, 2005) indicating no impact on the hatching time for both chemicals. In contrast, 560 µg/L of another alkylphenol, 4-tert-pentyl phenol, has been reported to increase hatching time in fathead minnow (Panter et al., 2006). Hatching time was not included in the zebrafish embryo test proposed by Schulte and Nagel (1994). Hatching time was also included as endpoint in the larvae hatching and survival test on the synthetic musks (Paper I) although by the use of a median value of hatching time based on 20 individuals obtained from manual observation.

Reduced pigmentation at 48 hpf was another sublethal endpoint with high sensitivity to extracts from oil platform effluents (Paper III). In six of the extracts this endpoint was the most sensitive. In other studies (Paper I & II), pigmentation decreases were not recorded. However, the assessment was performed in another, maybe less sensitive way in these studies. Reduced pigmentation has been reported in fish embryos after exposure to different types of chemicals. In embryos of African catfish (*Clarias gariepinus*) exposed to copper and cadmium, pigmentation reduction was recorded (Nguyen and Janssen, 2002). Weak pigmentation is also reported when exposing zebrafish embryos to the carrier solvents acetone, ethanol and DMSO (Hallare *et al.,* 2006). Reineke et al. (2006) found missing pigmentation to be the most sensitive nonlethal malformation in zebrafish embryos exposed to brominated phenoles. A mixture of alkylphenoles has been reported to result in decreased pigmentation in zebrafish embryos, whereas nonylphenol alone did not cause pigmentation effects in concentrations

up to 500 µg/L (Jörgenson, 2005). Nagel (2002) reported reduced pigmentation in zebrafish embryos after exposure to several anilines, phenols (including p-tert-butylphenol) and amines.

Decreased heart rate has been recorded as a sensitive response in testing of various chemicals including pharmaceuticals (Schulte and Nagel, 1994; Milan *et al.,* 2003). Heart rate was the most sensitive endpoint in zebrafish, *X. tropicalis* and moor frog in the studies of synthetic musks (Paper I & II). Further, heart rates were decreased in TBBPA-exposed zebrafish and *X. tropicalis* embryos (Paper II). The dose responses were very similar in all three species although *X. tropicalis* was less sensitive in this response when exposed to MK. In tests of platform effluents declines in heart rate were detected in several extract samples (Paper III). In high concentrations, the decline in heart rate was often observed in connection with oedemas or absence of circulation, suggesting more severe impact on heart and vascular system. Effects on heart rate have been reported in other studies after exposure to chemicals. In 3-day-old zebrafish exposed to process chemicals used in the oil refinery industry, a concentration-dependent decrease in heart rate was observed (Roseth *et al.,* 1996). Further, in Baltic herring exposed to different oil types during early development, decrease in heart rate was a major effect (Linden, 1978). Heart rate in zebrafish seems to respond very similar to the human heart to pharmaceutical exposure. In a test of 23 drugs known to cause repolarization in humans, 22 showed decreased heart rate in zebrafish embryos tested at 48 hpf (Milan *et al.,* 2003). This demonstrates the opportunity for the use of zebrafish embryos in drug screening or monitoring of effluents with high concentrations of certain pharmaceuticals.

Spontaneous movement was affected in zebrafish exposed to high concentrations of MK, MX and TBBPA (Paper I & II) as well as one effluent extract (Paper III). In all these tests, this endpoint was assessed as "yes/no" under a 30-s period. In the following tests of platform effluent extract, the number of movements was counted during 1 min. This showed no difference compared with the control group, but this fact is probably more related to other composition of the tested extract than to limitations in the way this endpoint was measured. The positive control mixture showed decline in movements in two of three tests.

Lethal endpoints include coagulation, absence of somites, no extension of tail, and lack of heart beat (Nagel, 2002). All these endpoints are serious enough to cause mortality even though embryos are not actually dead at the observation time, except for coagulation. These endpoints were used together in calculations of mortality even though the major observed mortal indication was embryos that were totally coagulated.

Sensitivity of method

The LOEC values of the synthetic musk substances recorded by the use of the embryo test are low compared with other reported tests. The LOECs in the present study were 10 µg/L for MK and 33 µg/L for both MX and AHTN, whereas HHCB-exposed embryos were unaffected even in the highest tested concentration,

1000 µg/L. The larvae hatching and survival test were as sensitive for MX, but not for the other tested musk substances. Reported toxicity tests on aquatic organisms such as *Daphnia magna*, photoluminescent bacteria, algae, rainbow trout (*Oncorhynchus mykiss*), *Xenopus laevis*, bluegill sunfish (*Lepomis macrochicus*) and early life-stage fathead minnow and *Nitocra spinipes*, the lowest LOECs were 100, 100, 77 and 140 µg/L for MK, MX, AHTN and HHCB respectively (Schramm *et al.,* 1996; Tas *et al.,* 1997; Chou & Dietrich, 1999; Balk & Ford, 1999; Breitholtz, Wollenberger & Dinan, 2003). In a larvae development test of the copepode *Acartia tonsa*, the LOECs were recorded to be 66, 26 and 59 µg/L for MK, AHTN and HHCB, respectively (Wollenberger *et al.,* 2003). These LOEC values are in the range of the recorded in the zebrafish embryo method, except for HHCB, implying that this is a relatively sensitive method, at least in tests of this type of chemicals. All three species tested with TBBPA gave a LOEC of 1000 µg/L, where oedema, absent circulation and mortality were the main findings. There are only a few toxicity studies reported on water exposure of TBBPA. In mysids (*Mysidopsis bahia*) a 96-h LC50 concentration of 860 µg/L has been reported (Goodman *et al.,* 1988). This substance has been detected in aquatic organisms (Morris *et al.,* 2004), sediment and sewage sludge (Sellström and Jansson, 1995) although concentrations in natural water are often below limit of detection (Kuiper *et al.,* 2007). Thus, there seems to be low risk for an acute toxic impact caused by water exposure to TBBPA, which suggests exposure through other sources. The tests of the synthetic musk compounds revealed LOEC values in the range of what have been recorded by chemical measurements in contaminated natural waters (Hahn, 1993; Paxéus, 1996; Heberer, Gramer & Stan*.,* 1999) which implies further attention.

Metamorphosis

Xenopus laevis has been the major amphibian species used to detect thyroid disrupting chemicals (Opitz *et al.,* 2005; Degitz *et al.,* 2005; Goleman, Carr & Anderson, 2002; Tietge *et al.,* 2005; Jagnytsch *et al.,* 2006). The use of *Xenopus tropicalis* as a test species to study the metamorphosis process in tadpoles required some modification of the experimental protocol suggested for *X. laevis*. Although the time for control *X. tropicalis* tadpoles to develop from stage 51 to 56 has been reported to be 28 days (Mitzui *et al.,* 2006), raising tadpoles at our laboratory was approximately 14 days from stage 51 to 58. The optimal temperature of culturing *X. tropicalis* is reported to be 26°C (Fort *et al.,* 2004c) which was the temperature used in this thesis as well. This partly explains a faster development as compared *with X. laevis* which is normally kept at lower temperatures (Opitz *et al.,* 2005). Further, the developmental rate has been reported to be higher in *X. tropicalis* during the first days after fertilization even when cultured at the same temperature as *X. laevis* (Fort *et al.,* 2004c). In the metamorphosis studies of PTU (Paper IV) and PBDEs (Paper V), median stage for controls was 57 and 58, respectively, after 14 days of exposure starting at stage 51, which corresponds to the stage obtained in 21-day studies starting at stage 51 using *X. laevis* (Degitz *et al.,* 2005; Opitz *et al.,* 2006; Jagnytsch *et al.,* 2006). The shorter duration time of metamorphosis offer considerable advantages to the use of *X. tropicalis*. The main endpoint in the metamorphosis studies on *X. laevis* has been the developmental stage. In addition,

apical endpoints such as hind limb length, body length, biochemical measurements corresponding to concentrations of thyroid hormones (Opitz *et al.,* 2006) and histopathological evaluation of thyroid gland (Degitz *et al.,* 2005; Tietge *et al.,* 2005; Opitz *et al.,* 2006) have been applied to increase the sensitivity and to further explore the mechanism of action. This thesis shows that some of these endpoints are applicable in *X. tropicalis* as well and contribute to the understanding of thyroid disruption mechanisms. The increased sensitivity by adding histological measurements on thyroid gland after PTU exposure (Paper IV) and the recorded effects on hind limb length and developmental stage with no effects on thyroid gland after PBDE exposure (Paper V) exemplify the need for measuring both apical and histological endpoints in these assays.

Apical endpoints

Some easily measured apical characteristics in tadpoles are influenced by thyroid hormone. The developmental stages (Nieuwkoop & Faber, 1994) which correspond to the overall developmental progress in *X. laevis* are interrupted after exposure to certain thyroid hormone inhibitors (Opitz *et al.,* 2006). The growth of the hind limb is thyroid hormone dependent as well as the regression of the tail (Tata, 2006) which occur late in the metamorphosis process. In addition, measurements corresponding to the tadpole health and growth independent of thyroid hormones such as body weight and body length are important to include to distinguishing between decreased developmental rate caused by toxic impact or as a result of thyroid disruption. In the study of tadpoles exposed to PTU (Paper IV), the hind limb length as well as developmental stage was clearly reduced in the highest tested concentration, 75 mg/L. Further, body weight and body length did not differ from other groups in the study and mortality was not recorded in any groups. These findings indicate that the decrease in the rate of metamorphosis process is due to a disruption of the thyroid system. The well-known mechanism of action of PTU (Shiroozu, 1983) as well as the recorded impact on thyroid gland histology supports this conclusion. In the study of tadpoles exposed to PBDEs (Paper V), mortality in the highest groups of both congeners revealed that the exposure to the lower concentrations was close to be toxic even though no mortality was recorded in these groups. In the second highest concentration of BDE-99, the tadpoles were in a lower developmental stage than controls at the same time as the hind limb length were shorter. Further, the body weight and body length remained unaffected, which suggests some kind of disruption of the thyroid disruption. This was however not confirmed by the thyroid gland histology. In tadpoles exposed to the second highest concentration of BDE-47, hind limb length was reduced although developmental stage was unaffected. In this group, body length and body weight were also reduced. This combination of responses, as well as the absence of effects in the thyroid gland histology measurements, suggests that the reduction in apical responses is more likely caused by a decline in overall growth than by a thyroid hormone disruption.

Histological endpoints

There are several mechanisms for chemical interference on thyroid hormone pathway, including inhibition of 5'-deiodinase, induction of hepatic microsomal

enzymes such as phenol sulfotransferase and UDP-glucuronyl transferase as well as disruption of thyroid hormone synthesis or secretion (Capen, 1997). Each of these mechanisms can reduce concentrations of circulating thyroid hormones. As a result, secretion of thyroid stimulating hormone (TSH) from the pituitary gland increases, which may cause structural changes such as hypertrophy, hyperplasia and neoplasia in the thyroid gland (Capen, 1997). These facts suggest that measurements of hormone concentrations as well as histopathological evaluation of the thyroid gland could add valuable information. O'Connor et al. (1999) stated that histopathological change of the thyroid gland is the ultimate sign of a true thyroid toxicant. Histological examination of the thyroid gland has been shown to be a sensitive parameter in several studies concerning thyroid disruption (Goleman, Carr & Anderson, 2002; Degitz *et al.,* 2005; Tietge *et al.,* 2005; Opitz *et al.,* 2006; van der Ven *et al.,* 2006). In the present thesis, thyroid histology was performed using simple length and area measurements on digital photographs. The largest thyroid gland cross section area on tadpoles exposed to PTU (Paper IV) showed a clear dose response relationship where all exposed groups had significantly larger cross-sectional areas than controls. This endpoint was the most sensitive and is comparable to a thyroid gland weight measurement; both reflect the overall size of the thyroid gland. O'Connor (1999) found the thyroid weight to be a sensitive endpoint to detect thyroid toxicants in rats. Follicular epithelial cell height showed a similar dose response relationship, where tadpoles in all PTU concentrations except in the lowest, 2 mg/L, had increased cell height. These findings, in combination with absence of effects on apical characteristics such as hind limb length, suggests that in *X. tropicalis*, compensatory regulation of the thyroid gland can prevent metamorphosis retardation in exposure to lower concentrations of PTU. A similar outcome has been reported for *X. laevis*, where perchlorate effects were recorded by histopathology measurements in lower concentrations than those detected by measuring apical developmental parameters (Tietge *et al.,* 2005). The increase in size of the whole thyroid gland and the increase in epithelial cell height can be explained by an increase in TSH levels as a result of feedback mechanism due to low circulating levels of thyroid hormones. As a response to the higher TSH levels, the thyroid follicular epithelial cells increase the activity of thyroid hormone production with resulting hyperplasia and hypertrophy (Capen, 1996). Dietary exposure of tadpoles to BDE-47 and -99 resulted in no effects either on thyroid cross section area or epithelial cell height, suggesting other mechanisms than for PTU of the recorded decreases in hind limb length and developmental stage in BDE-99 exposed tadpoles. These recordings are in agreement to Balch et al. (2006) where no effects in the cellular architecture of the thyroid gland, including the maximal area, were recorded on *X. laevis* tadpoles dietary exposed to the PBDE mixture DE-71. However, increased thyroid follicular epithelial cell height and colloid depletion have been recorded in rats exposed to DE-71 (Stoker *et al.,* 2004). These results point to differences between classes of animals in the outcome of PBDE exposure.

Conclusions

The zebrafish embryo assay is a powerful and robust tool which could be used to provide background information for risk assessment of chemicals. Moreover, some studied endpoints can contribute to the understanding of the mode of action of specific chemicals. Further, the simplicity of the assay and short duration makes it suitable for screening of large numbers of environmentally relevant chemicals or samples.

Xenopus tropicalis can be considered as a complementary species to *X. laevis* for evaluation of thyroid disrupting chemicals due to similarity in response between the two species.

In both assays, the option of individual exposure limits the number of test organisms, reduces interactions between individuals, requires only small quantities of chemicals, and can provide high statistical power.

Future perspectives

Further exploration of *X. tropicalis* as a candidate for evaluation of chemicals interfering with thyroid hormone endocrinology through different modes of action is needed.

Further development of the zebrafish embryo method by modern technologies such as microinjection should be conducted.

Including behavioural studies that can disclose exposure to chemicals, i. e. interfering with development of nervous system should be made.

Further validation of the principle of individual exposure is needed in order to optimize the assays and thereby also statistical power

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