Novel *in vitro, ex vivo* and *in vivo* assays elucidating the effects of endocrine disrupting compounds (EDCs) on thyroid hormone action

Merijn Schriks

Promotor:	Prof. dr. ir. I.M.C.M. Rietjens Hoogleraar in de Toxicologie Wageningen Universiteit
Co-promotoren:	Dr. A.J. Murk Universitair Hoofddocent Sectie Toxicologie Wageningen Universiteit
	Dr. J.D. Furlow Associate Professor Section of Neurobiology, Physiology and Behavior University of California, Davis (USA)
Promotiecommissie:	Prof. dr. A. Brouwer Vrije Universiteit Amsterdam
	Prof. dr. M. Scheffer Wageningen Universiteit
	Prof. dr. ir. T.J. Visser Erasmus Universiteit Rotterdam
	Dr. B.J. Blaauboer Institute for Risk Assessment Sciences, Utrecht

Dit onderzoek is uitgevoerd binnen de onderzoekschool WIMEK/SENSE.

Novel *in vitro*, *ex vivo* and *in vivo* assays elucidating the effects of endocrine disrupting compounds (EDCs) on thyroid hormone action

Merijn Schriks

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op woensdag 4 oktober 2006
des namiddags te half twee in de Aula.

The research described in this thesis was carried out at the Department of Toxicology, Wageningen University, The Netherlands.

Title Novel *in vitro*, *ex vivo* and *in vivo* assays elucidating the effects of endocrine disrupting compounds (EDCs) on thyroid hormone action.

Author Merijn Schriks
Thesis Wageningen University, Wageningen, the Netherlands (2006)
With abstract, with references, with summary in Dutch.

ISBN 90-8504-484-7

"I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

SIR ISAAC NEWTON (1642-1727)

Table of contents

1

General introduction, objectives and outline

1.1 Thyroid hormones

The thyroid gland was described for the first time in detail by Vesalius in the 16th century. Around 1656 Thomas Wharton named the organ thyroid gland, derived from the Greek word thyreos (meaning shield). In humans the gland is positioned in front of the shield-shaped cartilage of the larynx. The thyroid gland produces from iodine and the amino acid tyrosine, thyroid hormone (TH). Either deficient or excessive production of THs may lead to serious diseases. The first description of thyroid disease consisted of abnormal enlargement of the thyroid gland (goiter) in humans, recognised around 3000 B.C. As a remedy, it was recommended to eat seaweed, burned sponges (iodine) or dried deer thyroids (TH). Nowadays is has been well established that the occurrence of the goiter syndrome is ascribed to iodine deficiency. Fetal or maternal iodine deficiency (hyperthyroidism) may lead to development of a child with cretinism, which causes serious retardation of mental and physical development (Legrand, 1996).

It was not until 1896, that Fugen Bauman discovered that an iodine containing compound could be extracted from thyroid glands. This iodine containing hormone, (3,3',5,5'-tetraiodo-L-thyronine; thyroxine; T_4; Figure 1) was first isolated and crystallized by Edward Calvin Kendell in 1915. It took researchers another 40 years before the second form of TH (3,3',5-triiodo-L-thyronine; T_3; Figure 1), was identified by J. Gross and Pitt-Rivers.

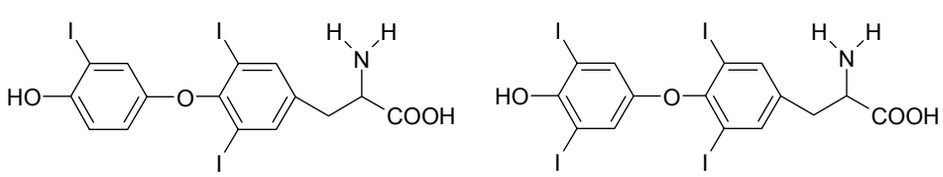


Figure 1. 3,3',5-triiodo-L-thyronine (T_3) (left) and 3,3',5,5'-tetraiodo-L-thyronine (T_4) (right)

This milestone discovery led to significant new findings in terms of primary effects of thyroid hormones in the last 30 years. In humans, THs are essential for the development of tissues (e.g. brain, heart, kidney, skeletal muscle and ear) (Brouwer et al., 1998; Hauser et al., 1998) and energy metabolism (Kadenbach et al., 1995; Nelson et al., 1995, Henneman, 1986). The synthesis of thyroid hormones

starts on the rough endoplasmatic reticulum, where formation of a large globular glycoprotein occurs. This so-called thyroglobulin contains tyrosine residues that can be iodinated and than coupled to form T_4. Release of THs by the thyroid gland is stimulated by thyrotropin (TSH) which is secreted by the anterior pituitary, which in turn is influenced by thyrotropin-releasing hormone (TRH) secreted by the hypothalamus (Figure 2).

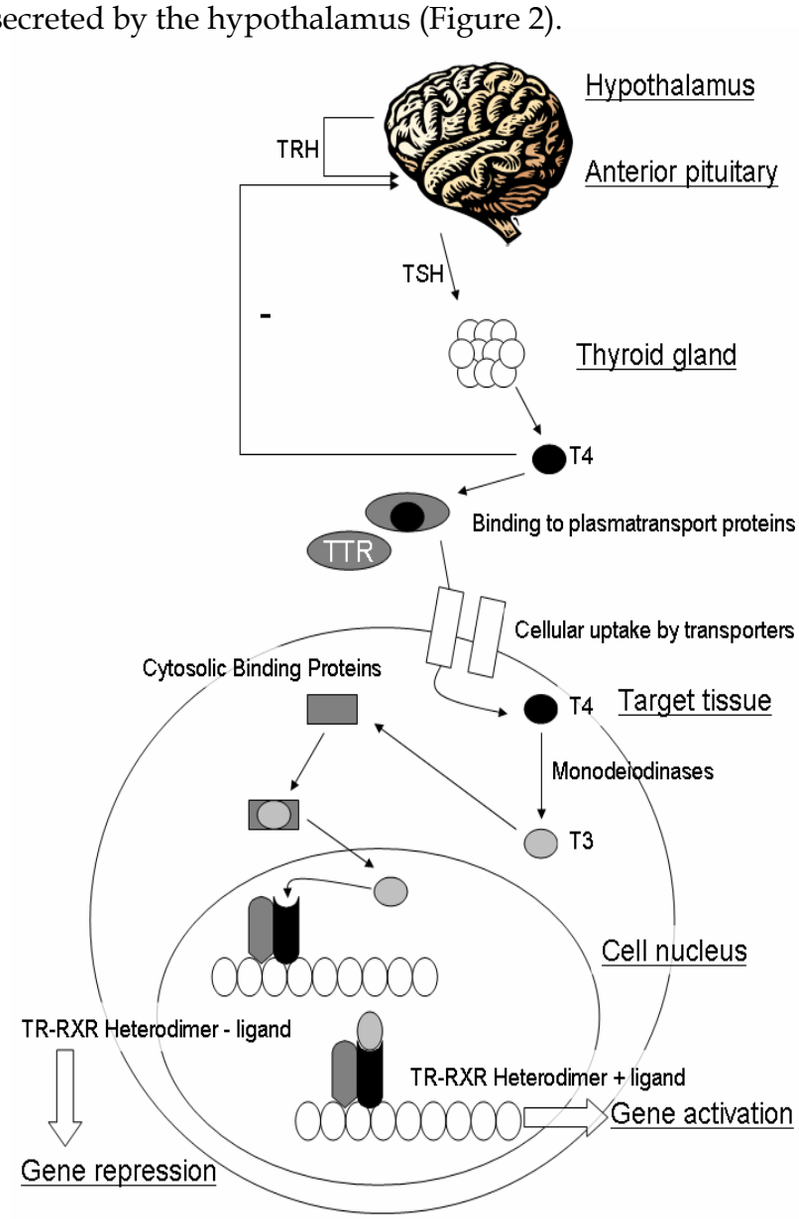


Figure 2. Regulation of the thyroid hormone axis at multiple levels of organisation. TRH, Thyrotropin Releasing Hormone; TSH, Thyroid Stimulating Hormone; T_4, 3,3',5,5'-tetraiodo-L-thyronine; TTR, TransThyRetin; T_3, 3,3',5-triiodo-L-thyronine; TR, Thyroid hormone receptor; RXR, Retinoid-X Receptor. Minus indicates a negative feedback loop. Reproduced with modifications from Hormones, Brain and Behavior (Elsevier, 2002).

At physiological pH, THs are poorly soluble in water and once secreted, they are bound to plasma proteins; the most important being TransThyRetin (TTR), albumin and thyroxin binding globulin (TBG). Only TBG appears to be fully selective for thyroid hormone transport alone (Robbins and Bartalena, 1986). Unlike humans, ungulates, carnivores, fish and amphibians do not express TBG (Larsson et al., 1985). TTR is not only important for transport of THs, but also for co-transportation of retinol binding protein (RBP) and mainly synthesized in the liver (Dickson et al., 1985). TH transport proteins not only function as TH plasma carriers, but also maintain a stable pool of THs from which the active free hormones are released for uptake by target cells (Robbins and Bartalena, 1986). THs have several metabolic fates and once secreted by the thyroid gland their biological activity is determined by 1) the peripheral concentration of the THs (T_3 and T_4), 2) the activities of iodothyronine deiodinases type I/II (D1/D2) and type III (D3), and 3) the presence of thyroid hormone transporters in cell membranes, which facilitate the uptake of T_3 and T_4 (Friesema et al., 2005). D1 and D2 catalyze mainly outer ring or 5'-deiodination (5'D) and thus are responsible for the generation of T_3. D3 catalyzes inner ring or 5-deioniadation (5D), a process that results in the degradation of both T_3 and T_4 to inactive metabolites (Henneman, 1986). For about 25 years it was assumed that cellular uptake of THs was a simple process of diffusion (Robbins, 1997). This hypothesis was based on the assumption that lipophilic THs were translocated by diffusion through the lipid-rich cellular membrane. After an initial publication about the saturable and energy-dependent transport of T_3 and T_4 into rat hepatocytes (Rao et al., 1976; Krenning et al., 1978; Docter et al., 1978), a whole series of reports confirmed energy (ATP)- and Na+-dependent carrier-mediated transport of THs into a variety of cells from different species. An extensive review on TH transport into different cell types and organs was published in 2001 (Hennemann et al., 2001).

1.2 Thyroid hormone action, a closer look

Within the cell, THs are transported to the nucleus by cytosolic binding proteins (CTHBPs) (Barsano and Groot, 1983; Cheng, 1991) (Figure 2). These proteins are non-specific and also serve other roles within the cytoplasm. Few studies have been carried out on CTHBPs, although it has been suggested that they play a role as binders of intracellular free THs, or as storage proteins to buffer cellular TH levels (Ashizawa and Chen, 1992; Shi et al., 2002). Furthermore, it is believed that they play a role in the non-genomic action of THs (Davis and Davis, 1996). The non-genomic action of THs is independent of nuclear receptors for the ligands. The biological effects of THs are believed to be mediated through the high specific thyroid hormone receptors (TRs). These receptors are highly conserved throughout evolution. *Xenopus laevis* TRα, for example, is >95 % and >91 % conserved in its DNA and ligand-binding domains respectively, when compared to human TRα. For TRβ these numbers yield an even higher homology, namely >96 % and >94 % respectively (Yaoita et al., 1990). In humans the action of T_3 is mediated via four functional receptors, TRα1, TRβ1,TRβ2 and TRβ3 encoded by two genes designated α- and β- c-erbA (Sap et al., 1986; Weinberger et al., 1986). TRα and TRβ within species share a high degree of similarity in their DNA-binding domain (DBD) and ligand binding domain (LBD), however the N-terminal region of these proteins is unrelated (Wu and Koenig, 2000). The result of these similarities is that both TRα and TRβ bind T_3 and T_4. However, various physiological functions are differentially regulated by each receptor. For example, treatment of *Xenopus laevis* tadpoles with the specific TRβ agonist GC-1 strongly induces loss of TRβ dominated larval tissues (gills, tail), whereas TRα rich tissues (brain) are not affected to a significant level (Furlow et al., 2004). Furthermore, TRα appears to play a more dominant role in TH control of the heart rate and body temperature of mice (Johansson et al., 1999; Wikstrom et al., 1998), whereas development of the inner ear appears to require TRβ (Forrest et al. 1996). From these studies, it is clear that TRα and TRβ are differentially involved in the control of developmental, endocrine and metabolic processes. T_3 binds both human TRα1

and TRβ1 with near-equal affinity (Chiellini et al., 1998). Dietrich and co-workers utilized quantitative structure-activity studies of thyroid hormone analogues in order to reveal the binding prerequisites of T_3 to TRs (Dietrich et al., 1977). Crucial for binding is the 4'-hydroxyl group of the ligand, acting as a hydrogen bond donor and acceptor. A hydrophobic substituent in the 3'-postion (iodine) increases the activity whereas hydrophilic substituents markedly decrease the affinity. The TRs have a lower affinity for TH analogues with substituents smaller than iodine. Furthermore, the affinity of the receptor for the ligand is decreased by the presence of a 5'-substituent larger than a hydrogen atom. The size of substituents in the 3-, 5 and 3'-positions are less restricted. The diphenylether oxygen atom is buried within the hydrophobic core of the ligand and not involved in TR binding. The carboxyl anion on the 1-position is important for TR affinity, most probably due to electrostatic interactions with a charged amino acid in the TR. Removal of the amino group on the side chain increases however the affinity slightly. TRs can bind specific DNA sequences called thyroid hormone response elements (TREs). Unliganded TRs recruit so-called co-repressors such as N-CoR and SMRT, which via interaction with conserved hydrophobic sequences, causes the gene to be silent (Koenig et al., 1998; Horlein et al., 1995). The best TRE binding heterodimers are formed between RXRs (retinoid-X receptors) and TRs (Wu and Koenig, 2000; 2001). These heterodimers not only bind to TREs but also regulate the transcription of TH response genes (Puzianowska et al., 1997). When T_3 binds to the TR-RXR heterodimer co-repressors are released and the complex disintegrates into monomers. Next, the ligand-bound TR monomer heterodimerizes with the RXR and binds again to the same TRE. Due to a conformational change within the ligand binding domain it now recruits co-activators such as N-CoR or SRC-1 (Koenig et al., 1998; Onate et al., 1995). This complex enhances gene transcription by opening chromatin situated close to the TR-regulated gene enabling RNA polymerase II to reach target DNA.

1.3 Role of thyroid hormones in amphibian development

The first causative relationship between tadpole metamorphosis and thyroid hormones was made by the German scientist Friedrich Gudernatsch (Gudernatsch, 1912). Tadpoles fed with macerated equine thyroid glands underwent precocious metamorphosis, and tadpoles with a removed thyroid gland were permanently arrested in their larval stage. The active component T_3 is now known to be the primary hormone responsible for amphibian metamorphosis. Although T_3 is required for initiation and completion of amphibian metamorphosis, other hormones are also involved (Hayes, 2000). Together with TH they form the hypothalamus-pituitary-thyroid (HPT) axis (Figure 3).

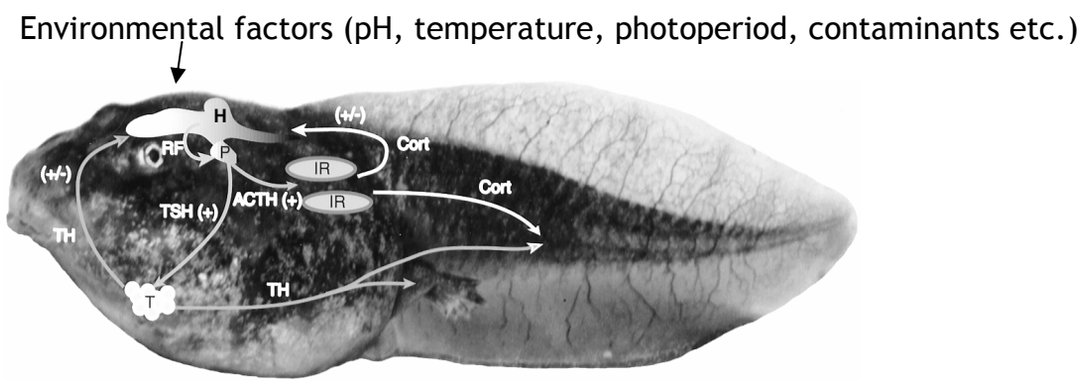


Figure 3. Thyroid hormone system controlling tadpole metamorphosis. H, hypothalamus; ACTH, adreno-corticotropic hormone; Cort, corticoids; IR, interrenal gland; P, pituitary gland; RF, releasing factor; T, thyroid gland; TH, thyroid hormone; TSH, thyroid-stimulating hormone. Pluses indicate a stimulatory effect and minuses a negative feedback.

During tadpole development, environmental signals (e.g. temperature, light, stress) are received by the hypothalamus and hormonally translated into stimulatory or inhibitory signals to the pituitary. The hypothalamus releases thyrotropin releasing hormone (TRH) which stimulates the pituitary lactotropes to produce prolactin, which in turn decreases levels of TRs in target tissues, thus antagonizing the action of TH. The hypothalamus also releases corticotropin

releasing hormone (CRH) which stimulates the release of both thyroid hormone stimulating hormone (TSH) and adrenocorticotrophic hormone (ACTH) from the pituitary thyrotropes and the corticotropes respectively (Dodd and Dodd, 1976; Kaltenbach, 1996; Shi, 2000). TSH stimulates the synthesis of TH and its release from the thyroid gland. ACTH stimulates the production of corticosterone in the interrenal glands. During amphibian metamorphosis, free swimming, aquatic herbivorous or filter-feeding larvae are transformed into terrestrial, air breathing, carnivorous adults. In order to accomplish this, three primary morphological changes occur during metamorphosis: 1) resorption or regression of tissue and/or organ systems (e.g. gills, tail); 2) remodelling of larval tissues and organ systems to an adult form (e.g. liver, intestine); 3) development of new tissues and organs (e.g. lungs, limbs) (Shi, 2000) (Table 1).

Table 1. Morphological and biochemical responses to thyroid hormone during amphibian metamorphosis (based on Tata, 1998; Shi, 2000).

Tissue	Morphological responses	Biochemical responses
Brain	Re-structuring, growth, differentiation, cell proliferation and apoptosis	Cell division, apoptosis and protein synthesis
Liver	Re-structuring, functional differentiation	Induction of urea cycle enzyme, larval to adult haemoglobin gene switching
Eye	Re-positioning, lens structure	Visual pigment transformation
Skin	Re-structuring, skin granular gland formation, keratinisation and hardening, apoptosis	Induction of collagen and keratin, induction of collagenase
Limb bud, lung,	*De novo* formation of bone, skin, muscle, nerves	Cell proliferation and differentiation
Tails, gills	Complete regression	Programmed cell death, induction and activation of lytic enzymes (caspases)
Pancreas, intestine	Major tissue restructuring	Reprogramming of phenotype, induction of proteases
Immune system	Re-distribution of cell populations	Altered immune-system and appearance of new immuno competent components
Muscle	Growth and differentiation; apoptosis	Induction of myosin heavy chain

Furthermore, various biochemical events are associated with amphibian metamorphosis (Table 1). Moreover, during metamorphosis a transition of waste-nitrogen excretion takes place. Tadpoles like most fishes, are ammonotelic thus excreting waste-nitrogen in the form of ammonia. Adult frogs on the other hand are ureotelic, excreting urea, like most terrestrial vertebrates. During metamorphosis, the liver develops the required enzymes to create urea from carbon hydroxide and ammonia (Weber, 1967). Etkin (1968) defined metamorphosis into three distinct phases: premetamorposis, prometamorphosis and metamorphic climax. Premetamorphosis refers to embryonic and early larval development under low concentration of THs. During prometamorphosis, cells of the thyroid gland grow, become secretory and release THs in the bloodstream (Figure 4). Limb bud development takes place and as an earliest response to TH, TRβ transcripts are formed (Shi, 2000). The synthesis of TRβ mRNA accelerates dramatically as metamorphosis commences (Figure 4).

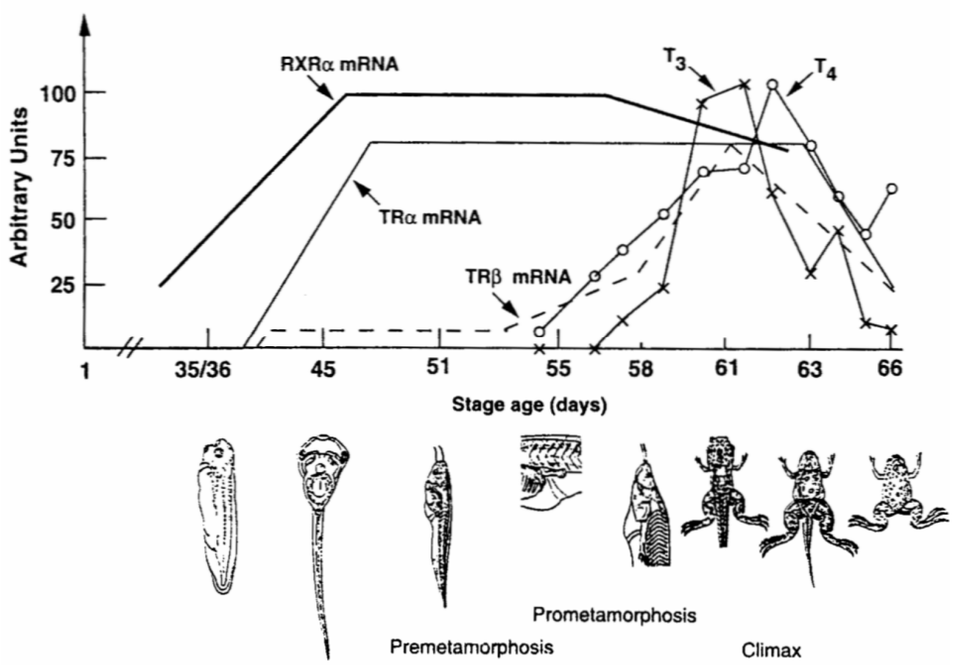


Figure 4. Differential expression of TRα, TRβ and RXRα during *Xenopus laevis* development. TH levels during development are in also indicated (Leloup and Buscaglia, 1977). The mRNA levels for TRs and RXRα are based on published data (Yaoita et al., 1990; Kawahara et al., 1991; Wong and Shi, 1995).

Induction of TRβ renders a tissue to become more competent in responding to THs. This autoinduction of TRβ by TH itself plays a significant role in the acceleration of metamorphosis toward the last developmental stage called metamorphic climax (Dent, 1988; Galton, 1988). A surge of TH triggers final processes in metamorphosis such as forelimb development and complete resorption of the tail. Following metamorphic climax TH levels decrease (Leloup and Buscaglia, 1977). As described, an important process during amphibian metamorphosis is the loss of tissues that are redundant in the adult animal. An important example is the tadpole tail, that is resorbed by a molecular and biochemical event called apoptosis (programmed cell death) (Yoshizato et al., 1993; Ishizuya-Oka et al., 1996; Shi et al., 1998; Gemar et al., 2001; Estabel et al., 2003). The tail is genetically predetermined to undergo apoptosis, and the onset of this process requires only little amounts of TH. This is illustrated in an experiment by Schwind (1933) where an extra tailtip was transplanted to the trunk region of a tadpole. The extra transplanted tailtip appeared not to be protected against degeneration, thus illustrating an organ-specific response of TH. During tail resorption, specific degradative enzymes are regionally upregulated. These enzymes, called caspases, play an important role for the resorption of the tadpole tail (Shi et al., 2000). Other functions of TH during amphibian development include keratinisation of the skin to conserve water and minimize the risk of mechanical injury for the terrestrial adult (Duellman & Trueb, 1986; Shi et al., 2000; Miller et al., 1998). From an evolutionary point of view, amphibians are phylogenetically separated from other vertebrate groups. However, specific aspects of TH signalling are conserved at morphological and molecular levels. Amphibian metamorphosis is developmentally relative equivalent to postembryonic organogenesis in mammals. Thus, the conserved nature of the TH axis provides a possibility for the use of amphibians as a model for other vertebrate species.

1.4 Thyroid hormone disruption by environmental contaminants

During the late 1980s, Theo Colborn a US scientist working for the WWF, put together different observations from wildlife and laboratory studies to what is now known as the "endocrine disruptor hypothesis" (Fairly et al., 1996; Colborn et al., 1996). An endocrine disruptor has been defined as an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle (EDSTAC, 1998). Till now, the majority of endocrine disruptor research has focused on interference of compounds with the sex hormone homeostasis, resulting in effects on sexual differentiation and reproduction. Less attention has been paid on disruption of the thyroid system, despite the fact that many species of birds and fish in the USA and Canadian Great Lakes and other water bodies around the world suffer unusual thyroid gland development and concentrations of circulating thyroid hormones (Colborn, 2002). As a downside from many technological breakthroughs since World War II, many chemicals have been released into the environment. Because most regulatory focus was on carcinogenic and teratogenic properties of compounds, most of them have not been tested for possible effects on thyroid hormone homeostasis. However, the thyroid gland is a relatively robust organ in adults, normally able to compensate for moderate disruption of the TH homeostasis. On the other hand, a distinction should be made in the case of unborn infants. Because thyroid hormones play such a crucial role in the development of the foetus, prenatal exposure to thyroid hormone disrupting compounds may have the most deleterious effects, particularly on the developing brain. This underlines the importance of the window of vulnerability.

The mechanism of action of thyroid hormone disrupting compounds is obscure. Brouwer and co-workers published an extensive review on the many mechanisms how so-called Polyhalogenated Aromatic Hydrocarbons (PHAHs) interfere with the thyroid hormone homeostasis (Brouwer et al. 1998). In summary, PHAHs

have been shown to interfere with 1) plasma TH levels; 2) thyroid gland function and morphology; 3) TH metabolism; 4) TH plasma transport; 5) TH nuclear receptor binding. Interference of PHAHs with no. 1 through 4 has also recently been reviewed (Janosek et al., 2005; Brouwer 1998), but interference of PHAHs with TH nuclear receptor binding (TRs) has only recently gained more attention (Zoeller et al., 2005). Compared to the estrogen receptor (ER), very little is known regarding the ability of compounds to interact with the TR. Now, several reports show that a broad range of environmental contaminants can interfere with TH binding to TRs and may produce complex effects on TH signalling. However, the pocket were the ligand binds to the TR has been shown by crystallography to be internal and provide a tight fit for T_3, and much less for T_4 and/or other TH analogues (McGrath et al., 1994). It has previously been hypothesized that the occurrence of environmental chemicals to bind to the TR is likely to be very rare (Brucker-Davis, 1998). Indeed, some progress in the production of selective TR (ant)agonists has been made, but till now amiodarone (low affinity), GC-1 and NH-3 are the only three recognized pharmaceutical compounds with capacity to bind the TR (Beeren et al., 1996; Yoshihara et al., 2003; Furlow et al., 2004; Lim et al., 2002). These compounds have pharmaceutical interest since T_3 is a potent serum cholesterol lowering agent (Shi et al., 2001), but the downsides of T_3 are various side-effects such as tachycardia and increased risk of cardiac arrhythmia (Klein et al., 2001). The application of GC-1 in hypothyroid rats indeed selectively decreased plasma lipids and thyroid-stimulating hormone secretions without increasing the heart rate (Trost et al., 2000; Baxter et al., 2004). As for environmental contaminants Marsh and co-workers showed specific binding of synthesized brominated diphenylethers (BDEs) to isolated human TRα and TRβ. Although the tested compounds had a high structural resemblance to T_3, the binding potency to TRα and TRβ was still roughly 500 and 150 times less than T_3, respectively. As described before binding affinity of environmental contaminants to the TR is generally low, but according to a recent review by Zoeller (2005) other

interactions of environmental contaminants with the TR-mediated cascade of events may be of greater importance (Figure 5).

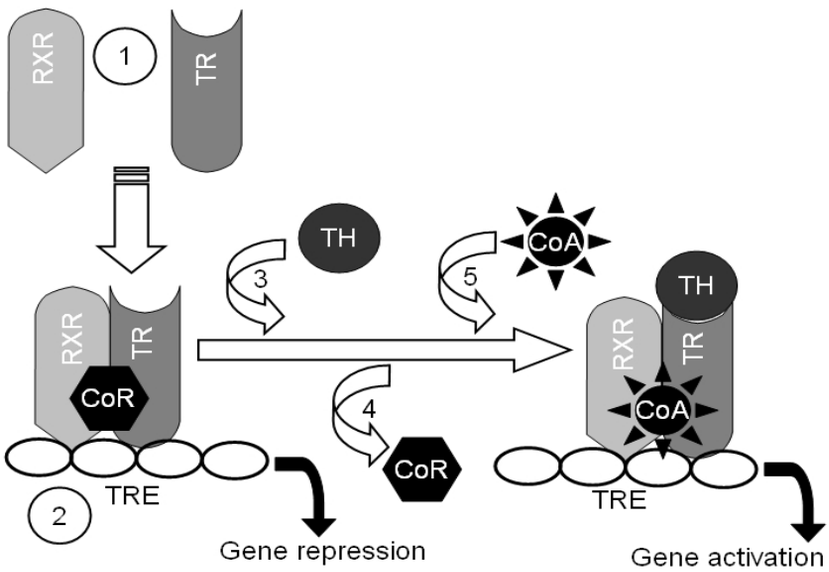


Figure 5. Anticipated sites at which substances can interfere with TH signalling on the molecular level. This action may occur at 1) Heterodimerization of the Thyroid hormone Receptor (TR) with Retinoid X Receptors (RXRs); 2) binding of the TR-RXR heterodimer to a Thyroid hormone Response Element (TRE); 3) Binding of thyroid hormone (TH) to a TR-RXR heterodimer; 4) Release of corepressors (CoR); 5) recruitment of coactivators (CoA). Reproduced with modifications from Zoeller et al., 2005.

Such interactions may occur at the level of the interaction of TRs with various co-factors such as N-CoR or SRC-1. In addition, compounds may cause the TR to exhibit a different affinity for its DNA binding domain. Moreover, in a recent study of Miyazaki and co-workers the influence of PCB on TR binding to the TRE was studied. It appeared that the TR-RXR heterodimer complex was partially dissociated from the TRE in the presence of PCB, leading to a suppression of transcription (Miyazaki et al., 2004). Finally, genomic effects beyond traditional TH mediated induction of transcription can be expected. Xenobiotic peroxidase proliferators (PPs), such as phtalates, bind to so-called peroxisome proliferator-activated receptor α (PPARα), and induce liver activities (e.g. malic enzyme) classically considered to be thyroid hormone dependent. This hepatic

thyromimetic activity of PPs is not mediated by binding to the TR (Hertz et al., 1996; Hertz et al., 1991).

1.5 Assays for thyroid hormone disruption

In 1996, the US Environmental Protection agency (EPA) formed the Endocrine Disruptor Screening and Advisory Comity (EDSTAC) to provide guidance and how to design testing programs to identify Endocrine Disrupting Compounds (EDCs). In its final report, EDSTAC recommended a two-tiered approach (Tier 1: screening; Tier 2: testing) for identification of these compounds. The primary objective of Tier 1 screening is to rapidly detect the potential of a compound to interfere with the TH signalling without the use of experimental animals. At the present there are a number of Tier 1 tests available, but none of them have been validated according to the standards of the Organisation for Economic Co-operation and Development (OECD), though there are possibilities. *In vitro* receptor binding studies can be used as potential screen for chemicals that bind to TRs (Marsh et al., 1998; Cheek et al., 1999) or to plasma transport proteins such as transthyretin (TTR) (Meerts et al., 2000; Cheek et al., 1999 Yamauchi et al., 2003). One problem with binding assays is that they cannot differentiate between agonists and antagonists. Furthermore these model systems cover only a very small part of the hypothalamus-pituitary-thyroid (HPT) axis and effects on the cellular transduction cascade involved in TR binding are not taken into account. Alternative assays have been developed that are able differentiate between agonistic or antagonistic action of compounds are systems in which a specific TR is transfected into a cell line along with a reporter gene, typically coding for luciferase, β-galactosidase or green fluorescent protein (Yamada-Okabe et al., 2005; Sugiyama et al., 2005).

Another significant disadvantage of *in vitro* (Tier 1) testing is the lack of metabolic capability. It is possible that the metabolites of some chemicals, and not the parent compound, are responsible for potential effects. For example, bisphenol-A (BPA)

appears to be a TR-antagonist (Moriyama et al., 2002), but the polyhalogenated derivatives are agonists (Kitamura et al., 2002).

To address biotransformation of compounds Tier 2 tests are required to more specifically determine and characterize the thyroid hormone disrupting effects using specific endpoints in an experimental animal model. To test whether a compound disrupts the HPT axis, currently a limited number of bioassays are available. EDSTAC recommends the following *in vivo* models: the adult male rat (to detect thyroid modulating compounds), the Japanese quail (TH-binding protein action) (Ishihara et al., 2003) and a Xenopus metamorphosis assay (XEMA-assay). Except for the latter assay none of the tests have been validated for guideline purposes. In the adult male rat assay a combination of hormone measurements with histopathology of the thyroid gland are required endpoints, while measuring of T_3 levels is optional. The XEMA assay, which is meant to provide an indication of mammalian thyroid effects, has been specifically designed to reveal both anti-thyroidal as well as thyromimetic activities of environmental contaminants (Opitz et al., 2005). The primary endpoint for the XEMA assay is the developmental stage to which *Xenopus laevis* tadpoles develop during 28 days of exposure to compounds. However, this assay does not provide any insight into the mechanism of action of compounds. Furthermore, determination of the primary site of effect and/or identification of causal effects versus downstream subsequent resulting effects is not possible.

In summary, there are no *in vitro* systems that have been validated for use as a thyroid screen. In part, this may be because the general focus has been on the ability to affect thyroid function, not thyroid hormone action. However, as more chemicals are found to influence TR function, there is a need to develop and validate new *in vitro* screens. Such assays may also serve as useful tools to understand and elucidate the mechanisms by which compounds affect TR-mediated gene regulation. Furthermore, to take events such as absorption, distribution, metabolism, elimination (ADME) into account, the development and validation of a TR-specific *in vivo* model is required. For reasons mention under

section 1.3, amphibian postembryonic development would serve as an excellent parameter to address this issue. Finally, *in vitro* and *in vivo* bioassay responses need to be compared, to be able to assess *in vivo* effects based on the *in vitro* results.

1.6 The South African clawed toad

The test species used for the *in vivo* and *ex vivo* experiments in the present thesis is the South African clawed toad, *Xenopus laevis*. It is one of the most widely used laboratory animals in the world and belongs to the family Pipidae, order Anura. Animals in the order Pipidae are solely aquatic, with their tongues completely attached to the palatum. *Xenopus laevis* (from Greek meaning "strange foot") is the most widely distributed species of the fourteen species in the Xenopus genera and is native to Southern Africa. *Xenopus laevis* offers several advantages as a test species for laboratory studies. It is relatively easy to maintain and breed in captivity and is widely accepted test animal for developmental studies in the scientific community (ASTM, 1991). Its embryonic development has been described in great detail (Nieuwkoop and Faber, 1975), and the species is relatively easy to obtain from commercial suppliers. Reproduction can be induced using human chorionic gonadotrophic injections, and the larvae can be bred in large numbers to selected stages. There is a lot of literature available on the biochemical and metabolic control of THs in this species (EPA, 2002).

1.7 Outline of the thesis

The objectives of the research described in this thesis are threefold:

1. Development and validation of *in vitro* and *in vivo* assays for disruption of thyroid hormone action on multiple levels of biological organization (Figure 6).
2. Elucidation of the potential mechanisms of TR-mediated disruption.
3. Advice whether predictions can be made based on *in vitro* studies for *in vivo* disruption of thyroid hormone action.

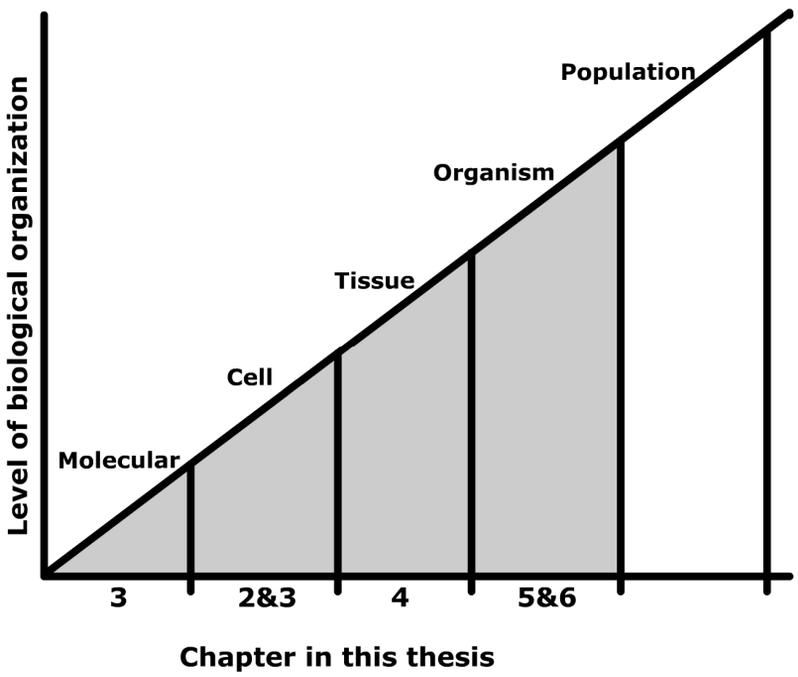


Figure 6. The multiple levels of biological organization used in this thesis for testing of thyroid hormone disrupting effects. The numbers indicated on the horizontal axis indicate the chapters that deal with a specific level of biological organization.

To address the first objective, several assays were developed and validated on multiple levels of biological organisation (Figure 6). Chapter 2 describes the application of an earlier developed *in vitro* bioassay at the cellular level, termed the T-screen (Gutleb et al., 2005). Apart from optimization of the *in vitro* bioassay, several Brominated Flame Retardants (BFRs), including brominated diphenylethers were tested for effects on thyroid hormone action. The application of a Thyroid hormone Receptor (TR) isoform selective bioassay on the cellular level forms the basis of chapter 3. Until now, this bioassay has been exclusively used for the application of pharmaceutical compounds (Furlow et al., 2004) and for the present thesis work a selection of environmental relevant compounds derived from chapter 2 and other studies were tested. Chapter 4 explores the use of *Xenopus laevis* tail tips in organ culture to quantify the effects of two BFRs on T_3-induced tail resorption. Chapter 5 describes the use of an *in vivo* proliferation assay using one-week old *Xenopus laevis* tadpoles to examine if two model

compounds used in chapter 2 & 3 & 4 elicit comparable responses *in vivo*. In order to determine differential effects of the compounds in tissues, responses were quantified in the brain and rostral head region. To explore how a thyroid hormone disrupting compound (triphenyltin) affects a higher level of biological organisation, the effect of triphenyltin on *Xenopus laevis* tadpole behavior was studied with the aid of the Multispecies Freshwater Biomonitor (MFB). The outcome of this study is presented in chapter 6.

The second objective of the present thesis is discussed in chapter 7 and accomplished by combining the assay responses which will enhance the insight in the potential mechanisms of thyroid hormone disruption. Chapter 7 also aims to meet the third objective by comparing the assays applied within the scope of this thesis. Finally, the perspectives for the use of the mechanism based *in vitro* and *in vivo* assays in toxicological research and the possible implications of thyroid hormone disrupting compounds are discussed.

References

American Society for Testing and Materials, A. 1991. Standard guide for conducting the frog embryo teratogenesis assay-Xenopus. Annual book of ASTM standards. p 1-11.

Ashizawa, K., Cheng, S. Y., 1992. Regulation of Thyroid-Hormone Receptor-Mediated Transcription by a Cytosol Protein. **P Natl Acad Sci USA 89**, 9277-9281.

Barsano, C. P., Groot, L. 1986. Nuclear cytoplasmic interrelationships. Oppenheimer J, Samuels H, editors. New York: Academic Press. 139– 177 p.

Baxter, J. D., Webb, P., Grover, G., Scanlan, T. S., 2004. Selective activation of thyroid hormone signaling pathways by GC-1: a new approach to controlling cholesterol and body weight. **Trends Endocrinol Metab 15**, 154-157.

Beeren van, H. C., Bakker, O., Wiersinga, W. M., 1996. Structure-function relationship of the inhibition of the 3,5,3'-triiodothyronine binding to the alpha1- and beta1-thyroid hormone receptor by amiodarone analogs. **Endocrinology 137**, 2807-2814.

Brouwer, A., Morse, D. C., Lans, M. C., Schuur, A. G., Murk, A. J., Klasson-Wehler, E., Bergman, A., Visser, T. J., 1998. Interactions of persistent environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. **Toxicol Ind Health 14**, 59-84.

Brucker-Davis, F., 1998. Effects of environmental synthetic chemicals on thyroid function. **Thyroid 8**, 827-856.

Cheek, A. O., Kow, K., Chen, J., McLachlan, J. A., 1999. Potential mechanisms of thyroid disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. **Environ Health Persp 107**, 273-278.

Cheng, S.-Y. 1991. Interaction of the thyroid hormone with cytosol proteins. Wu S-Y, editor. Boston: Blackwell Scientific Publications.

Chiellini, G., Apriletti, J. W., Yoshihara, H. A., Baxter, J. D., Ribeiro, R. C., Scanlan, T. S., 1998. A high-affinity subtype-selective agonist ligand for the thyroid hormone receptor. **Chem Biol 5**, 299-306.

Colborn, T., 2002. Clues from wildlife to create an assay for thyroid system disruption. **Environ Health Persp 110 Suppl 3**, 363-367.

Colborn, T., Myers, J. P., Dumanoski, D., 1996. Hormonal sabotage. **Nat His 105**, 42-49.

Davis, P. J., Davis, F. B., 1996. Nongenomic actions of thyroid hormone. **Thyroid 6**, 497-504.

Dent, J. N., 1988. Hormonal Interaction in Amphibian Metamorphosis. **Am Zool 28**, 297-308.

Dickson, P. W., Howlett, G. J., Schreiber, G., 1985. Rat Transthyretin (Prealbumin) - Molecular-Cloning, Nucleotide-Sequence, and Gene-Expression in Liver and Brain. **J Biol Chem 260**, 8214-8219.

Dietrich, S. W., Bolger, M. B., Kollman, P. A., Jorgensen, E. C., 1977. Thyroxine analogues. 23. Quantitative structure-activity correlation studies of *in vivo* and *in vitro* thyromimetic activities. **J Med Chem 20**, 863-880.

Docter, R., Krenning, E., Bernard, H., Visser, T., Henneman, G., 1978. Uptake of T_3 and T_4 by Cultured Rat-Liver Parenchymal-Cells. **Annales D Endocrinologie 39**, A44-a44.

Dodd, M., Dodd, J. 1976. The Biology of Metamorphosis. Lofts B, editor. New York: Academic Press. 467 - 599 p.

Duellman, E., Trueb, L. 1986. Biology of amphibians. London: The John Hopkins press, Ltd.

EDSTAC. 1998. Endocrine disruptor screening and testing advisory committee final report. http://www.epa.gov/scipoly/oscpendo/edspoverview/finalrpt.htm.

EPA, U. 2002. Revised draft detailed review paper for amphibian metamorphosis assay. Washington, D.C.

Estabel, J., Mercer, A., Konig, N., Exbrayat, J.-M., 2003. Programmed cell death in *Xenopus laevis* spinal cord, tail and other tissues, prior to, and during, metamorphosis. **Life Sci 73**, 3297-3306.

Etkin, W. 1968. Hormonal control of amphibian metamorphosis. Etkin W, Gilbert L, editors. New York: Appleton. 313 - 348 p.

Fairley, P., Colborn, T., Myers, J. P., 1996. Our stolen future: What pointers for industry? **Chem Week 158**, 55-56.

Forrest, D., Hanebuth, E., Smeyne, R. J., Everds, N., Stewart, C. L., Wehner, J. M., Curran, T., 1996. Recessive resistance to thyroid hormone in mice lacking thyroid hormone receptor beta: Evidence for tissue-specific modulation of receptor function. **Embo J 15**, 3006-3015.

Friesema, E. C. H., Jansen, J., Milici, C., Visser, T. J. 2005. Thyroid Hormone Transporters. Vitamins & Hormones: Academic Press. p 137.

Furlow, J. D., Yang, H. Y., Hsu, M., Lim, W., Ermio, D. J., Chiellini, G., Scanlan, T. S., 2004. Induction of larval tissue resorption in *Xenopus laevis* tadpoles by the thyroid hormone receptor agonist GC-1. **J Biol Chem 279**, 26555-26562.

Galton, V. A., 1988. The Role of Thyroid-Hormone in Amphibian Development. Am Zool 28, 309-318.

Gemar, K., Kiehl, D., Carlson, T. 2001. Contribution of apoptosis to tadpole metamorphosis. Pacific Lutheran University.

Gundernatsch, J., 1912. Feeding experiments on tadpoles I. The influence of specific organs given as food on growth and differentiation: a contribution to the knowledge or organs with intestinal secretion. **Arch. Entwicklungsmech. Org 35**, 457-483.

Hass, U., Dalgaard, M., Jarfelt, K., Kledal, T. S. A., 2004. Conceptual Framework for Testing and Assessment of Endocrine Disrupters as a basis for regulation of substances with endocrine disrupting properties. **TemaNord 555**.

Hauser, P., McMillin, J. M., Bhatara, V. S., 1998. Resistance to thyroid hormone: Implications for neurodevelopmental research on the effects of thyroid hormone disruptors. **Toxicol Ind Health 14**, 85-101.

Hayes, T. B. 2000. Endocrine disruption in amphibians. Sparling DW, Linder G, Bishop CA, editors. Pensacola: SETAC.

Henneman, G. 1986. Thyroid hormone metabolism. Henneman G, editor. New York: Marcel Dekker, Inc.

Hennemann, G., Docter, R., Friesema, E. C., de Jong, M., Krenning, E. P., Visser, T. J., 2001. Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. **Endocr Rev 22**, 451-476.

Hertz, R., Aurbach, R., Hashimoto, T., Bartana, J., 1991. Thyromimetic Effect of Peroxisomal Proliferators in Rat-Liver. **Biochem J 274**, 745-751.

Hertz, R., Nikodem, V., BenIshai, A., Berman, I., Bartana, J., 1996. Thyromimetic mode of action of peroxisome proliferators: Activation of 'malic' enzyme gene transcription. **Biochem J 319**, 241-248.

Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamel, Y., Soderstrom, M., Glass, C. K. and others. 1995. Ligand-Independent Repression by the Thyroid-Hormone Receptor-Mediated by a Nuclear Receptor Co-Repressor. **Nature 377**, 397-404.

Ishihara, A., Sawatsubashi, S., Yamauchi, K., 2003. Endocrine disrupting chemicals: interference of thyroid hormone binding to transthyretins and to thyroid hormone receptors. **Mol Cell Endocrinol 199**, 105-117.

Ishizuya-Oka, A., 1996. Apoptosis of larval cells durin amphibian metamorphosis. **Microsc Res Techniq 24**, 228-235.

Janosek, J., Hilscherova, K., Blaha, L., Holoubek, I., 2005. Environmental xenobiotics and nuclear receptors-Interactions, effects and *in vitro* assessment. **Toxicol in Vitro 20**, 18-37.

Johansson, C., Gothe, S., Forrest, D., Vennstrom, B., Thoren, P., 1999. Cardiovascular phenotype and temperature control in mice lacking thyroid hormone receptor-beta or both alpha(1) and beta. **Am J Physiol - Heart C 276**, H2006-H2012.

Kadenbach, B., Barth, J., Akgun, R., Freund, R., Linder, D., Possekel, S., 1995. Regulation of Mitochondrial Energy Generation in Health and Disease. **Biochim Biophys Acta 1271**, 103-109.

Kaltenbach, J. 1996. Endocrinology of Amphibian Metamorphosis. Gilbert LI, Tata JR, Atkinson BG, editors. New York: Academic Press. 403-431 p.

Kawahara, A., Baker, B. S., Tata, J. R., 1991. Developmental and Regional Expression of Thyroid-Hormone Receptor Genes during Xenopus Metamorphosis. **Development 112**, 933-943.

Kitamura, S., Jinno, N., Ohta, S., Kuroki, H., Fujimoto, N., 2002. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A **Biochem Biophys Res Commun 293**, 554-559.

Klein, I., Ojamaa, K., 2001. Mechanisms of disease: Thyroid hormone and the cardiovascular system. **New Engl J Med 344**, 501-509.

Koenig, R. J., 1998. Thyroid hormone receptor coactivators and corepressors. Thyroid 8, 703-713.

Krenning, E. P., Docter, R., Bernard, H. F., Visser, T. J., Hennemann, G., 1978. Active-Transport of Triiodothyronine (T_3) into Isolated Rat-Liver Cells. **Febs Letters 91**, 113-116.

Larsson, M., Pettersson, T., Carlstrom, A., 1985. Thyroid-Hormone Binding in Serum of 15 Vertebrate Species - Isolation of Thyroxine-Binding Globulin and Prealbumin Analogs. **Gen Comp Endocr 58**, 360-375.

Legrand, J. 1986. Thyroid hormone effects on growth and development. Henneman G, Henneman G, editors. 503-534 p.

Leloup, J., Buscaglia, M., 1977. Triiodothyronine, Hormone of Amphibian Metamorphosis. **Comptes Rendus Hebdomadaires Des Seances De L'Academie Des Sciences Serie D 284**, 2261-2263.

Lim, W., Nguyen, N.-H., Yang, H. Y., Scanlan, T. S., Furlow, J. D., 2002. A Thyroid Hormone Antagonist That Inhibits Thyroid Hormone Action *in Vivo*. **J Biol Chem** 277, 35664-35670.

Marsh, G., Bergman, A., Bladh, L.-G., Gillner, M., Jackobsson, E., 1998. Synthesis of p-Hydroxybromodiphenyl Ethers and binding to the thyroid receptor. **Organohalogen Compounds 37**, 305-309.

Mcgrath, M. E., Wagner, R. L., Apriletti, J. W., West, B. L., Ramalingam, V., Baxter, J. D., Fletterick, R. J., 1994. Preliminary Crystallographic Studies of the Ligand-Binding Domain of the Thyroid-Hormone Receptor Complexed with Triiodothyronine. **J Mol Biol** 237, 236-239.

Meerts, I. A., van Zanden, J. J., Luijks, E. A., van Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A., Brouwer, A., 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. **Toxicol Sci 56**, 95-104.

Miller, L. 1998. Hormone induced changes in keratin gene expression during amphibian metamorphosis. Metamorphosis. p 599-623.

Miyazaki, W., Iwasaki, T., Takeshita, A., Kuroda, Y., Koibuchi, N., 2004. Polychlorinated biphenyls suppress thyroid hormone receptor-mediated transcription through a novel mechanism. **J Biol Chem 279**, 18195-18202.

Moriyama, K., Tagami, T., Akamizu, T., Usui, T., Saijo, M., Kanamoto, N., Hataya, Y., Shimatsu, A., Kuzuya, H., Nakao, K., 2002. Thyroid hormone action is disrupted by bisphenol A as an antagonist. **J Clin Endocrinol Metab 87**, 5185-5190.

Nelson, B. D., Luciakova, K., Li, R. G., Betina, S., 1995. The Role of Thyroid-Hormone and Promoter Diversity in the Regulation of Nuclear-Encoded Mitochondrial Proteins. **Biochim Biophys Acta 1271**, 85-91.

Nieuwkoop, P. D., Faber, J. 1975. Normal table of *Xenopus laevis*. Amsterdam, The Netherlands: North Holland Publishing.

Onate, S. A., Tsai, S. Y., Tsai, M. J., Omalley, B. W., 1995. Sequence and Characterization of a Coactivator for the Steroid-Hormone Receptor Superfamily. **Science 270**, 1354-1357.

Opitz, R., Lutz, I., Nguyen, N. H., Scanlan, T. S., Kloas, W., 2005. Analysis of thyroid hormone receptor betaA mRNA expression in *Xenopus laevis* tadpoles as a means to detect agonism and antagonism of thyroid hormone action. **Toxicol Appl Pharmacol 24**, 653-664.

Puzianowska-Kuznicka, M., Damjanovski, S., Shi, Y. B., 1997. Both thyroid hormone and 9-cis retinoic acid receptors are required to efficiently mediate the effects of thyroid hormone on embryonic development and specific gene regulation in *Xenopus laevis*. **Mol Cell Biol 17**, 4738-4749.

Rao, G. S., Eckel, J., Rao, M. L., Breuer, H., 1976. Uptake of Thyroid-Hormone by Isolated Rat-Liver Cells. **Biochem Biophys Res Co 73**, 98-104.

Robbins, J., 1997. Cellular uptake of thyroid hormone. **Thyroid Int 4**, 4-7.

Robbins, J., Bartalena, L. 1986. Plasma transport of thyroid hormones. Hennemann G, editor. New York: Marcel Dekker Dekker, Inc.

Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H., Vennstrom, B., 1986. The C-Erb-a Protein Is a High-Affinity Receptor for Thyroid-Hormone. **Nature 324**, 635-640.

Schwind, J. L., 1933. Tissue specificity at the time of metamorphosis in frog larvae. **J Exp Zool 66**, 1-14.

Shi, Y.-B., Ritchie, J. W. A., Taylor, P. M., 2002. Complex regulation of thyroid hormone action: multiple opportunities for pharmacological intervention. **Pharmacol Therapeut 94**, 235-251.

Shi, Y. B. 2000. Amphibian metamorphosis - from morphology to molecular biology: John Wiley & Sons. 288 p.

Shi, Y. B., Su, Y., Li, Q., Damjanovski, S., 1998. Auto-regulation of thyroid hormone receptor genes during metamorphosis: roles in apoptosis and cell proliferation. **Int J Dev Biol 42**, 107-116.

Sugiyama, S.-i., Miyoshi, H., Yamauchi, K., 2005. Characteristics of a thyroid hormone responsive reporter gene transduced into a *Xenopus laevis* cell line using lentivirus vector. **Gen Comp Endocr 88**, 367-374.

Tata, J. R. 1998. Hormonal signaling and postembryonic development. Berlin: Springer.

Trost, S. U., Swanson, E., Gloss, B., Wang-Iverson, D. B., Zhang, H. J., Volodarsky, T., Grover, G. J., Baxter, J. D., Chiellini, G., Scanlan, T. S. and others. 2000. The thyroid hormone receptor-beta-selective agonist GC-1 differentially affects plasma lipids and cardiac activity. **Endocrinology 141**, 3057-3064.

Weber, R. 1967. Biochemistry of amphibian metamorphosis. Weber R, editor. New York: Academic press.

Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruol, D. J., Evans, R. M., 1986. The C-Erb-a Gene Encodes a Thyroid-Hormone Receptor. **Nature 324**, 641-646.

Wikstrom, L., Johansson, C., Salto, C., Barlow, C., Barros, A. C., Baas, F., Forrest, D., Thoren, P., Vennstrom, B., 1998. Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor alpha 1. **Embo J 17**, 455-461.

Wong, J. M., Shi, Y. B., 1995. Coordinated Regulation of and Transcriptional Activation by Xenopus Thyroid-Hormone and Retinoid-X Receptors. **J Biol Chem 270**, 18479-18483.

Wu, Y., Koenig, R. J., 2000. Gene regulation by thyroid hormone. Trends Endocrinol Metab 11, 207-211.

Wu, Y., Xu, B., Koenig, R. J., 2001. Thyroid hormone response element sequence and the recruitment of retinoid X receptors for thyroid hormone responsiveness. **J Biol Chem 276**, 3929-3936.

Yamada-Okabe, T., Satoh, Y., Yamada-Okabe, H., 2003. Thyroid hormone induces the epression of 1-1BB and activation of caspases in a thyroid hormone receptor-dependent manner. **Eur J Biochem / FEBS 270**, 3064-3073.

Yamauchi, K., Ishihara, A., Fukazawa, H., Terao, Y., 2003. Competitive interactions of chlorinated phenol compounds with 3,3',5-triiodothyronine binding to transthyretin: detection of possible thyroid-disrupting chemicals in environmental waste water. **Toxicol Appl Pharmacol 187**, 110-117.

Yaoita, Y., Shi, Y., Brown, D., 1990. *Xenopus laevis* α and β Thyroid Hormone Receptors. **P Natl Acad Sci USA 87**, 7090-7094.

Yoshihara, H. A. I., Apriletti, J. W., Baxter, J. D., Scanlan, T. S., 2003. Structural determinants of selective thyromimetics. **J Med Chem 46**, 3152-3161.

Yoshizato, K., Nishikawa, A., Izutsu, Y., Kaiho, M., 1993. Epidermal cells of the tail of an anuran larva are competent to transform into adult-type cells. **Zool Sci 10**, 183-187.

Zoeller, R. T., 2005. Environmental chemicals as thyroid hormone analogues: New studies indicate that thyroid hormone receptors are targets of industrial chemicals? **Mol Cell Endocr 242**, 10-15.

2

T-screen to quantify functional potentiating, antagonistic and thyroid hormone-like activities of polyhalogenated aromatic hydrocarbons (PHAHs)

Merijn Schriks[1], Cozmina M. Vrabie[1], Arno C. Gutleb[2], Elisabeth J. Faassen[1], Ivonne M.C.M Rietjens[1], Albertinka J. Murk[1]

[1]Division of Toxicology, Wageningen University, Wageningen, The Netherlands
[2]Dept. of production Animal Clinical Science, Norwegian School of Veterinary Science, Oslo, Norway

Toxicology in vitro 20 (2006) 490-498

Abstract

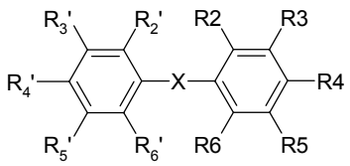
The present study investigates chemical thyroid hormone disruption at the level of Thyroid hormone Receptor (TR) functioning. To this end the (ant)agonistic action of a series of xenobiotics was tested in the newly developed T-screen. This assay makes use of a GH_3 rat pituitary cell line, that specifically proliferates when exposed to 3,3',5-triiodo-L-thyronine (T_3). The growth stimulatory effect is mediated via the TRs. (Ant)agonistic and potentiating action of compounds was studied in absence and presence of T_3 at its EC_{50} level (0.25 nM). The compounds tested included the specific TR antagonist amiodarone, as well as a series of brominated diphenylethers (BDEs), including specifically synthesized BDEs with a structural resemblance to 3,5-diiodo-L-thyronine (T_2), T_3 and 3,3',5,5'-tetraiodo-L-thyronine (T_4). The results obtained reveal that only BDE206 and amiodarone are specific antagonists. Interestingly some compounds which did not respond in the T-screen in absence of T_3, potentiated effects when tested in combination with T_3. This points at possibilities for disruption at the TR *in vivo*, where exposure generally occurs in presence of T_3. Altogether the results of the present study show that the newly developed T-screen can be used as a valuable tool for identification and quantification of compounds active in disturbing thyroid hormone homeostasis at the level of TR-functioning.

Introduction

Many persistent pollutants present in food chains and aquatic ecosystems have been shown to interfere with endocrine functions. In animals and man, these so-called endocrine disrupting chemicals (EDCs) can interfere with hormonal systems, and deviations at certain critical time periods may lead to significant consequences (Crofton, 2004). Most of the studies on EDCs have focused on interference with sex-hormone homeostasis, resulting in effects on sexual differentiation and reproduction (Colborn, 2002). However, in addition to (anti)-estrogenic, and (anti)-androgenic effects, also other important hormone systems can be affected. For example the thyroid hormone system, known to be important for development and energy metabolism, has been reported to be affected at several levels of homeostatic control (Legrand, 1986). Disturbances in thyroid hormone (TH) function and metabolism may lead to abnormal development, altered growth patterns, and a variety of physiological perturbations in mammals (Leung et al., 1990; Murk et al., 1998) as well as in fish, birds and amphibians (Besselink et al., 1996; Gutleb et al., 2000; Jung et al., 1997; Murk et al., 1994; Snodgrass et al., 2004; Veldhoen et al., 2001). As a result there is an increasing need for fast test systems that can be employed to detect and quantify potential thyroid hormone disrupting characteristics of persistent pollutants and their metabolites, but also of other existing as well as newly developed chemicals. A previous *in vitro* test for thyroid hormone disrupting activities was based on the capacity of xenobiotics to interfere with binding of T_4 to the plasma transport protein transthyretin (TTR). Several polyhalogenated aromatic hydrocarbons (PHAHs) including poly chlorinated biphenyls (PCBs) and poly brominated diphenylethers (PBDEs) and their hydroxylated metabolites were tested, as well as several known endocrine disruptors including bisphenol A (BPA) and some of its derivatives, were shown to be active in this TTR-T_4 displacement assay (Brouwer et al., 1986; Brouwer et al., 1990; Darnerud et al., 1996; Lans et al., 1993; Lans et al., 1994; Meerts et al., 2000). Recently we reported a new test method, called the T-screen, useful for *in vitro* detection of agonistic and antagonistic

properties of compounds at the level of the Thyroid hormone Receptor (TR) (Gutleb et al., 2005). The assay employs a rat pituitary tumor GH_3 cell line, the growth of which is dependent on the active thyroid hormone 3,3',5-triiodo-L-thryonine (T_3). The growth stimulatory effect of T_3 is mediated by well-characterized specific, high-affinity TRs that upon binding of THs bind to Thyroid hormone Responsive Elements (TREs) in the cell nucleus ultimately leading to gene expression (Hinkle and Kinsella, 1986). Interaction of xenobiotics with the TRs may result in agonistic effects on cell growth, whereas binding of antagonists to the TRs may result in inhibiting effects on T_3 mediated cell growth. Thus the T-screen provides possibilities to detect both agonists as well as antagonists at the level of TR-functioning. It has been generally accepted that structural resemblance of xenobiotics to thyroid hormones causes displacement of T_4 from TTR (Meerts et al., 2000). Since TR-mediated effects have not been studied yet, the objective of the present study was to investigate the structure requirements of xenobiotics to interfere with TR-related effects in the T-screen. To this end, some known T_3-specific (ant)agonists, but also a series of synthesized T_3-, T_2- and T_4-like xenobiotics (Table 1) were tested for their agonistic and antagonistic activity in the T-screen. The model compounds tested include brominated 4'-hydroxy diphenylethers synthesized previously as T_2-, T_3- or T_4-like models (Marsh et al., 1998), but also a series of brominated diphenylethers and tetrabromobisphenol A derivatives, demonstrated to be active in the T_4-displacement assay with TTR (Meerts et al., 2000). The T-screen was further optimized for fast and cheap screening of T_3-like activity. These optimizations include the replacement of alamarBlue™, used in the previous study (Gutleb et al., 2005) by the much cheaper resazurine, and the use of fetuin added as an additional growth factor to the medium to stimulate attachment, spreading and growth of the GH_3 rat pituitary cells when cultured in serum-free medium.

Table 1. Structures of the compounds used in the present study.



General structure compounds	X	R2	R3	R4	R5	R6	R2'	R3'	R4'	R5'	R6'
3,5-diiodo-L-thyronine (T_2)[a]	O	I	H	CH_2CHNH_2COOH	H	I	H	H	OH	H	H
3,3',5-triiodo-L-thyronine (T_3)[a]	O	I	H	CH_2CHNH_2COOH	H	I	H	I	OH	H	H
3,3',5,5'-tetraiodo-L-thyronine (T_4)[a]	O	I	H	CH_2CHNH_2COOH	H	I	H	I	OH	I	H
T_2-like BDE-OH (4'-hydroxy-2,4,6-tribromo diphenylether)	O	Br	H	Br	H	Br	H	H	OH	H	H
T_3-like BDE-OH (4'-hydroxy-2,3',4,6-tetrabromo diphenylether)	O	Br	H	Br	H	Br	H	Br	OH	H	H
T_4-like BDE-OH (4'-hydroxy-2,3',4,5',6-pentabromo diphenylether)	O	Br	Br	Br	H	Br	H	H	OH	Br	H
Brominated diphenylether69	O	Br	H	Br	H	Br	H	Br	H	H	H
Brominated diphenylether75	O	Br	H	Br	H	Br	H	H	Br	H	H
Brominated diphenylether127	O	H	Br	Br	Br	H	H	Br	H	Br	H
Brominated diphenylether185	O	Br	Br	Br	Br	Br	Br	H	H	Br	H
Brominated diphenylether206	O	Br	Br	Br	Br	Br	Br	Br	Br	Br	H
Bisphenol A (BPA)	C-$(CH_2)_2$	H	H	OH	H	H	H	H	OH	H	H
Tetrabromobisphenol A (TBBPA)	C-$(CH_2)_2$	H	Br	OH	Br	H	H	Br	OH	Br	H
Tetrachlorobisphenol A (TCBPA)	C-$(CH_2)_2$	H	Cl	OH	Cl	H	H	Cl	OH	Cl	H

Amiodarone and HBCD are presented in Figure 1, because they do not comply with the general structure.
[a]Numbering system used for thyroid hormones

Materials and methods

Chemicals

All chemicals were of > 98 % purity unless otherwise stated. Three hydroxylated brominated diphenylethers 4'-hydroxy-2,4,6-tribromodiphenylether, 4'-hydroxy-2,3',4,6-tetrabromodiphenylether, 4'-hydroxy-2,3',4,5',6-pentabromodiphenylether that are further abbreviated as a T_2-like BDE-OH, a T_3-like BDE-OH and a T_4-like BDE-OH, were a kind gift of Professor Åke Bergman and synthesized as described (Marsh et al., 1998) (at least 99 % pure). Tetrabromobisphenol A (TBBPA, 97 %), tetrachlorobisphenol A (TCBPA) and bisphenol A (BPA, 97 %) were obtained from Aldrich Chemical (Bornem, Belgium). 3,3',5-triiodo-L-thyronine, amiodarone (2-butyl-3-[3,5-diiodo-4-(β-diethylaminoethoxy)-benzoyl]benzofuran), fetuin (from fetal calf serum) and resazurine were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Dimethylsulfoxide (DMSO,

99.9 % pure) was of spectrometric grade and obtained from Acros Organics (New Jersey, USA). BDEs, hexabromocyclododecane (HBCD technical mixture, further abbreviated as HBCD TM) and hexabromocyclododecane (HBCD-γ) used for this study were kindly provided by Professor Åke Bergman (Stockholm University, Sweden) and also tested in the framework of the EU FIRE project, except for BDE69 which was obtained from Accustandard (New Haven, USA) (at least 99 % pure) and BDE75 which was synthesized as described (Marsh et al., 1998). 3,3',5-triiodo-L-thyronine stock solutions were prepared in DMSO and stored at –20 °C prior to use. All other compounds were dissolved in DMSO and kept in the dark at room temperature, except for resazurine, which was dissolved in phosphate buffered saline (PBS), aliquoted and stored at –20 °C. For completeness all tested compounds are presented in Table 1 and Figure 1.

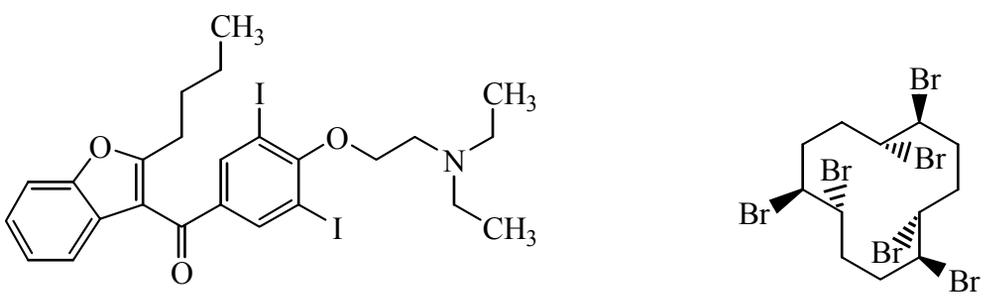


Figure 1. Structure of amiodarone (left) and HBCD-γ

Cell culture

Rat pituitary tumor GH_3 cells were cultured in a humid atmosphere at 37 °C and 95 % air / 5 % CO2 in Dulbecco's Modified Eagle's medium/ Ham's F12 (Gibco) supplemented with 10 % Fetal Calf Serum (Gibco-Invitrogen, Breda, The Netherlands) and 15 mM HEPES. Cells were sub-cultured once a week in new 25 cm^2 culture flasks (Greiner Bio-one, Alphen aan de Rijn, The Netherlands). Prior to the T-screen procedure cells were incubated for 48 hours in serum-free DMEM/F12 medium (PCM-medium) to deplete cells of thyroid hormones. In some cases fetuin (800 $\mu g.mL^{-1}$, indicated in legend when appropriate) was added to the serum free medium to promote attachment, spreading and growth of cells

(Fisher et al., 1958) . Serum free PCM medium was prepared as previously described (Gutleb et al., 2004; Gutleb et al., 2005; Sirbasku et al., 1991).

T-screen procedure

The T-screen was essentially carried out as described previously (Gutleb et al., 2005) with modifications. Briefly, on the day of exposure old serum free PCM medium was removed and cells were collected from the culture flask with the aid of a cell scraper (Corning, Schiphol-Rijk, The Netherlands), and plated at a density of 2500 cells/well on a transparent 96-well tissue culture plate (Greiner). Since cells were used in serum free conditions, scraping was applied for this procedure in order to prevent deleterious effects of trypsin. Exposure media were prepared on transparent 48-well tissue culture plates (Corning), by 200-250 times dilution in fresh PCM medium of stock solutions of the test chemicals (in DMSO). The final DMSO concentration was kept at ≤ 0.5 % (v/v) to prevent cytotoxic effects. In the case of antagonistic studies, PCM medium was supplemented with 3,3',5-triiodo-L-thyronine (T_3) at a final concentration of 0.25 nM T_3 in the well. This concentration was chosen because it corresponds with the EC_{50} of T_3 in the rat pituitary GH_3 cell system (Gutleb et al., 2005). GH_3 cells were exposed in triplicate and incubated for 96 hours with test chemicals at concentrations ranging from 0.1 μM to 10 μM. A complete standard curve with the biological active T_3 in the concentration range from 0.001 nM to 1000 nM and a vehicle control was included in each assay. This T_3 concentration range was chosen because it covers the active range of T_3 in the GH_3 cell system.

Resazurine cell proliferation assay

Cell proliferation was determined by measuring the total metabolic activity of GH_3 cells using the dye resazurine (O'Brien et al., 2000). This dye is non-radioactive, non-toxic and water soluble. Enzymes in the mitochondria of GH_3 cells reduce oxidized blue resazurine to the highly fluorescent pink complex resorufin. The fluorescence is a measure for the amount of viable cells present.

Following a 96 hours exposure as described above 8 µL, of a 400 µM resazurine solution in PBS (Gibco) was added to each well. After four hours of incubation in the dark (37 °C; 95 % air / 5 % CO2) fluorescence was measured at λex = 530 nm and λem = 590 nm on a Milli-pore Cytofluor 2350 Fluorescence Measurement System. Cell proliferation is expressed as a mean percentage of the maximum T_3 induced effect (set at 100 %).

BrdU-cell proliferation assay

A colorimetric immunoassay kit (Roche, Cat. No. 1647229) was used for quantification of cell proliferation on the basis of DNA cell content. This assay is based on the measurement of BrdU incorporation during DNA synthesis. For these experiments, cells were exposed to T_3 according to the T-screen protocol (see above). Following a 96 hours exposure, BrdU-labeled medium was added to the cells for an additional 4 hours. To prevent loss of labeled cells by washing, 96-wells plates were dried completely in a stove at 60 °C (1 h) prior to continuation of the assay, which was essentially mainly performed according to the protocol provided by the supplier. Cell proliferation was expressed as a mean percentage of the maximum T_3 induced effect (set at 100 %).

Data analysis

Curve fitting was performed using Life Science Workbench (LSW) Data Analysis Toolbox Version 1.1.1 (MDL Information Systems, San Leandro, CA, USA). The General sigmoidal curve with Hill slope (a to d) was selected as best fit model. Relative potencies of compounds compared to T_3 were calculated by dividing the EC_{50} of T_3 by the EC_{50} of the test compound. High repetition accuracy of the T-screen has been demonstrated earlier (Gutleb et al., 2005) and therefore a duplicate replication of each experiment with triplicate measurements was found to be sufficient.

Results

Validation of T-screen modifications

Fetuin addition resulted in a stimulation of GH_3 cell proliferation by 41 % and a slight shift in the EC_{50} of T_3 from 0.2 ± 0.03 nM in the absence to 0.1 ± 0.01 nM in the presence of fetuin (Figure 2). Thus, fetuin can be added to the T-screen whenever improved attachment and cell proliferation are required.

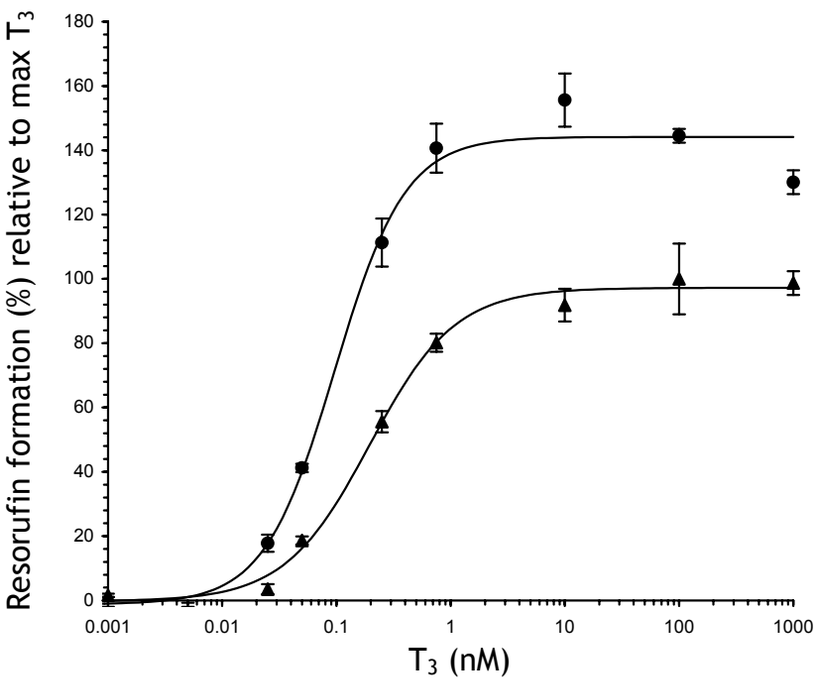


Figure 2. T_3 dependent rat GH_3 cell proliferation in the absence (triangles) and presence (circles) of fetuin. Cells were plated in presence of increasing concentrations of T_3 (2500 cells/ well) and maintained in serum free PCM medium for 96 h. Error bars were calculated as SD and data presented are means from triplicate datapoints and expressed as relative percentage of maximum cell proliferation from T_3 exposed cells (1000 nM) (without fetuin supplementation). Absolute numbers for control values were: without fetuin: 690 (solvent control) – 1611 (1000 nM T_3) relative fluorescence units (RFU); with fetuin: 800 (solvent control) – 2005 (1000 nM T_3) RFU.

To confirm that resazurine reduction was really reflecting cell proliferation, the T-screen was also performed with a BrdU assay measuring DNA replication to detect cell proliferation. The results obtained in the BrdU assay correspond to those obtained with the resazurine detection assay, with only a slight and un-significant shift in the EC_{50} of T_3 from 0.17 ± 0.04 nM to 0.14 ± 0.02 nM (Figure 3).

However, the standard deviation of the data points in the BrdU-assay is about 4-fold higher compared to that in the resazurine-assay. In T-screens performed with resazurin the intra-experimental variation varies from 1.2 % to 3 % in the solvent control and from 1.2 % to 4.9 % in the positive control (T_3 max) with an inter-experimental variation of 1.7 % (solvent) and 2.8 % (T_3 max).

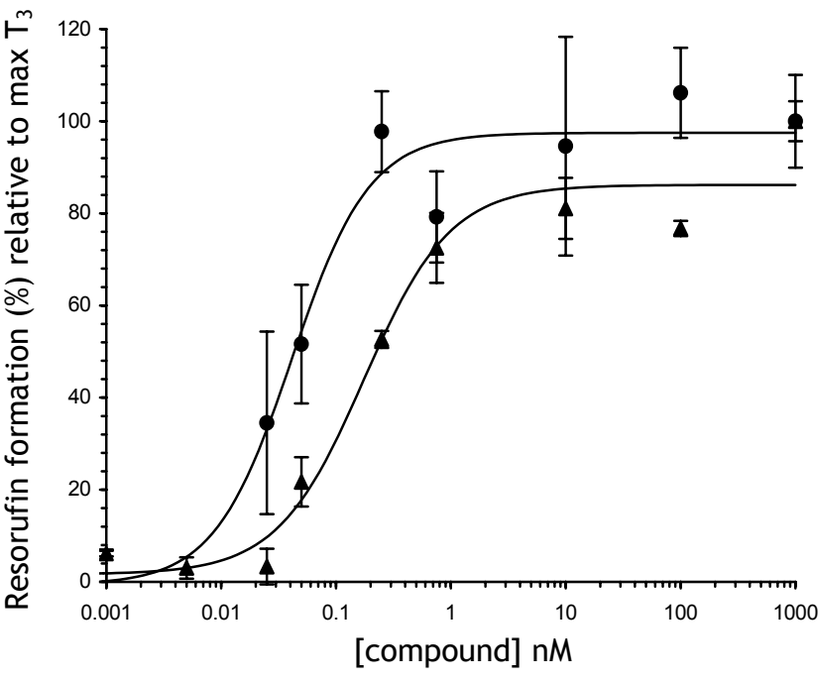


Figure 3. T_3 dependent rat GH_3 cell proliferation as measured with the resazurin-assay (triangles) and BrdU-assay (circles). Cells (2500 / well) were plated in presence of increasing concentrations of T_3 and maintained in serum free PCM medium for 96 h. Error bars were calculated as SD and data for both assays presented are means from triplicate data points and expressed as relative percentage of maximum cell proliferation from T_3 exposed cells (1000 nM). Absolute numbers for control values were: 4356 (solvent control) – 8791 (1000 nM T_3) RFU.

T-screen activity of T_2-, T_3- and T_4-like hydroxylated brominated diphenylethers

The EC_{50} for T_3-agonist action by the T_3-like BDE-OH is 214 ± 19 nM, which is three orders of magnitude higher than that of T_3 (EC_{50} = 0.21 ± 0.12 nM). The T_4-like BDE-OH does induce some agonistic activity with an EC_{50} that amounts to 1.42 ± 0.02 μM, which is 6-fold higher than the EC_{50} value for the T_3-like BDE-OH.

The T_2-like BDE-OH has no effect on GH_3 cell proliferation when tested up to 10 µM (Figure 4).

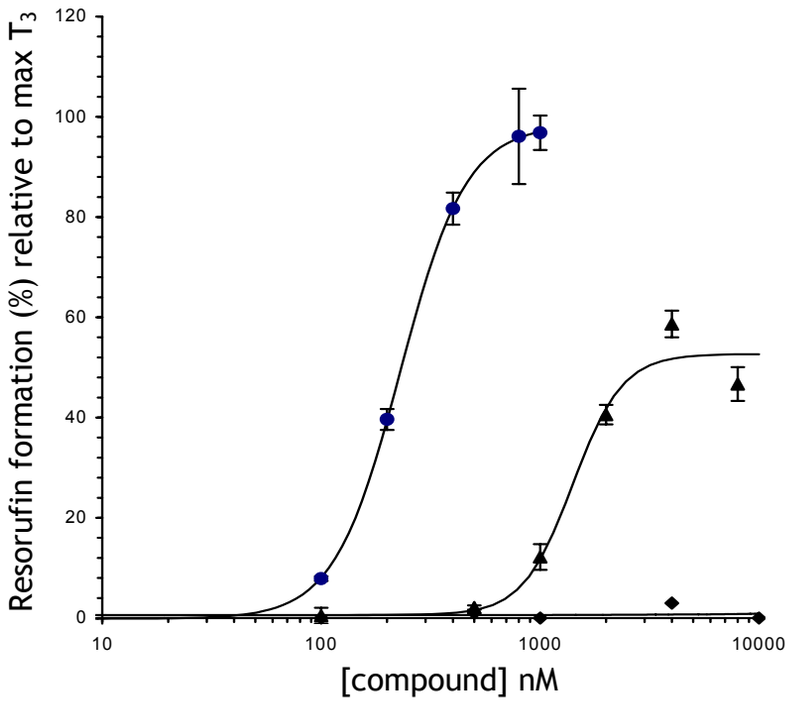


Figure 4. T-screen activity of T_2-like BDE-OH (diamonds), T_3-like BDE-OH (circles) and T_4-like BDE-OH (triangles). Cells were plated (2500 cells/ well) and exposed to compounds in serum free PCM medium for 96 h. Error bars were calculated as SD and data presented are means from triplicate data points and expressed as relative percentage of maximum cell proliferation from T_3 exposed cells (1000 nM). Absolute numbers for control values were: 3155 (solvent control) – 4998 (1000 nM T_3) RFU.

T-screen activity of xenobiotics in absence of T_3

Tested xenobiotics include the brominated diphenylethers BDE69, BDE75, BDE127, BDE185 and BDE206 as well as tetrabromobisphenol A (TBBPA), tetrachlorobisphenol A (TCBPA) and bisphenol A (BPA). Table 2 summarizes the EC_{50} values, relative potencies defined as the ratio of EC_{50} (T_3) / EC_{50} (compound), and maximal induction percentages for stimulation of proliferation of the GH_3 cells. Of the tested brominated diphenylethers only BDE69, BDE127 and BDE185 were able to stimulate cell proliferation to a marginal extent, and this was not statistically significant. TBBPA, TCBPA and BPA did not induce GH_3 cell proliferation at all.

Table 2. T-screen results of xenobiotics (see Table 1 for abbreviations of compounds) tested for agonism in the absence of exogenous T_3.

T-screen results				
Compound	Highest tested conc. (μM)	Max. induction (%)	EC_{50} (nM)	Relative potency[a]
T_4	0.1[b]	100[b]	0.65[b]	0.29[b]
T_3	1	100 ± 13	0.21 ± 0.12	1
T_2-like BDE-OH	10	n.d.	>10000	$>2*10^{-5}$
T_3-like BDE-OH	1	97 ± 6	228	$9*10^{-4}$
T_4-like BDE-OH	10	59 ± 7	1418	$2*10^{-4}$
BDE69	1	5 ± 1	>1000	$>2*10^{-4}$
BDE127	12.7	14 ± 6	9169[c]	$4*10^{-5}$
BDE185	12.4	11 ± 3	1090[c]	$3*10^{-4}$
TBBPA	100	n.a.	>100000	n.a.
TCBPA	100	n.a.	>100000	n.a.
BPA	1000	n.a.	>100000	n.a.
HBCD(TM)	1000	n.a.	>100000	n.a.
HBCD-γ	1000	n.a.	>100000	n.a.

n.a. = not active; no effects observed in the tested range
[a]Calculated as the ratio of EC_{50} (T_3)/EC_{50} (compound)
[b]Gutleb et al. (2005)
[c]Determined at EC_{10}

T-screen activity in the presence of T_3

In a final series of experiments the T-screen activity of the selected xenobiotics was tested in the presence of 0.25 nM T_3, the EC_{50} value. These conditions mimic the *in vivo* conditions best, where the compounds of concern never are present without thyroid hormones. This setup provides the possibility to detect antagonistic as well as synergistic or potentiating activities of the various model compounds. To validate the use of the assay for detection of antagonistic activities, first the model T_3 antagonist, amiodarone, was tested. This compound is reported to be more TR-specific than the generally applied diphenylhydantion (DPH) (Gingrich et al., 1985; Gutleb et al., 2005; Mann et al., 1983; Smith et al., 1984; Zemel et al., 1988). Amiodarone was able to completely antagonize GH_3 cell proliferation in the presence of 0.25 nM T_3 (Figure 5) at approximately 3 μM. The IC_{50} of amiodarone was 2.1 μM. At concentrations much higher than 3 μM amiodarone became cytotoxic.

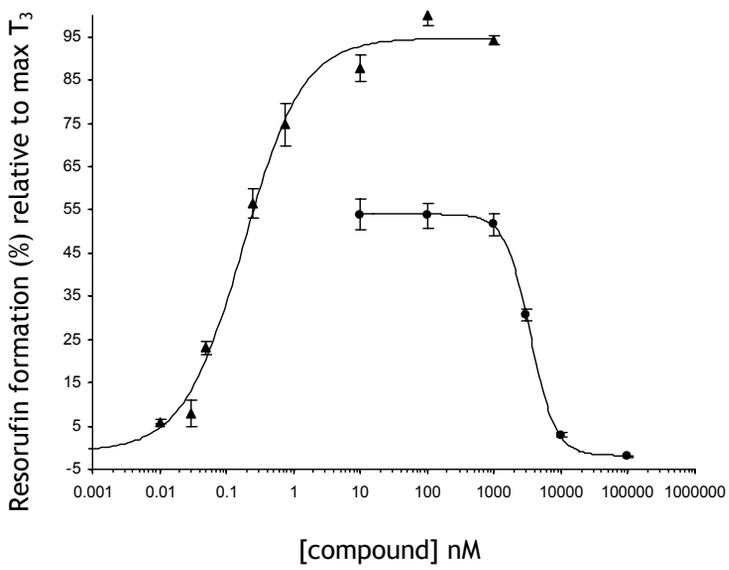


Figure 5. Agonistic action of T_3 on rat GH_3 cell proliferation (triangles) and antagonistic action of amiodarone in presence of 0.25 nM T_3 (circles). Cells were plated (2500 cells/ well) and exposed to amiodarone in serum free PCM medium for 96 h. Error bars were calculated as SD and data presented are means from triplicate data points and expressed as relative percentage of maximum cell proliferation from T_3 exposed cells (1000 nM). Absolute numbers for control values were: 3649 (solvent control) – 9148 (1000 nM T_3) RFU.

Of all the BDEs tested only one BDE acted as an antagonist, namely BDE206 with an IC_{50} of 449 nM, so much more potent than amiodarone (Figure 6).

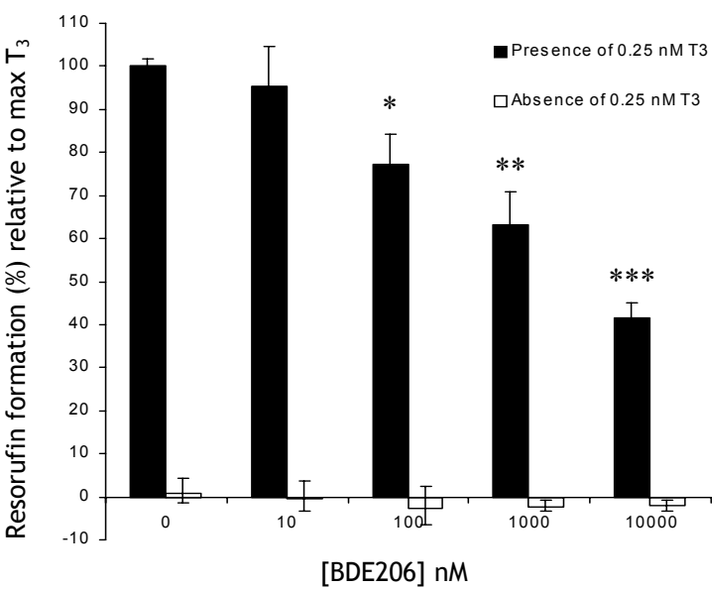


Figure 6. Effect of BDE206 in the presence and absence of 0.25 nM T_3 on rat GH_3 cell proliferation. Cells were plated (2500 cells/ well) and exposed to BDE206 in serum free PCM medium for 96 h. Error bars were calculated as SD and data presented are means from triplicate data points and expressed as percentage to the blanc which was set at 100 %. Absolute numbers for control values were: 3649 (solvent control) – 9148 (1000 nM T_3) RFU. * Significantly different from control ($p < 0.05$); ** $p < 0.01$; *** $p < 0.001$.

Three compounds acted potentiating in the presence of T_3 (0.25 nM), namely HBCD (TM), HBCD-γ and bisphenol A (Figure 7). Without addition of T_3 these compounds did not significantly induce cell proliferation in the T-screen (data not shown). Maximum potentiation of cell proliferation was induced at 1 μM test compound in the presence of 0.25 nM T_3. At higher concentrations these compounds started to become to be cytotoxic.

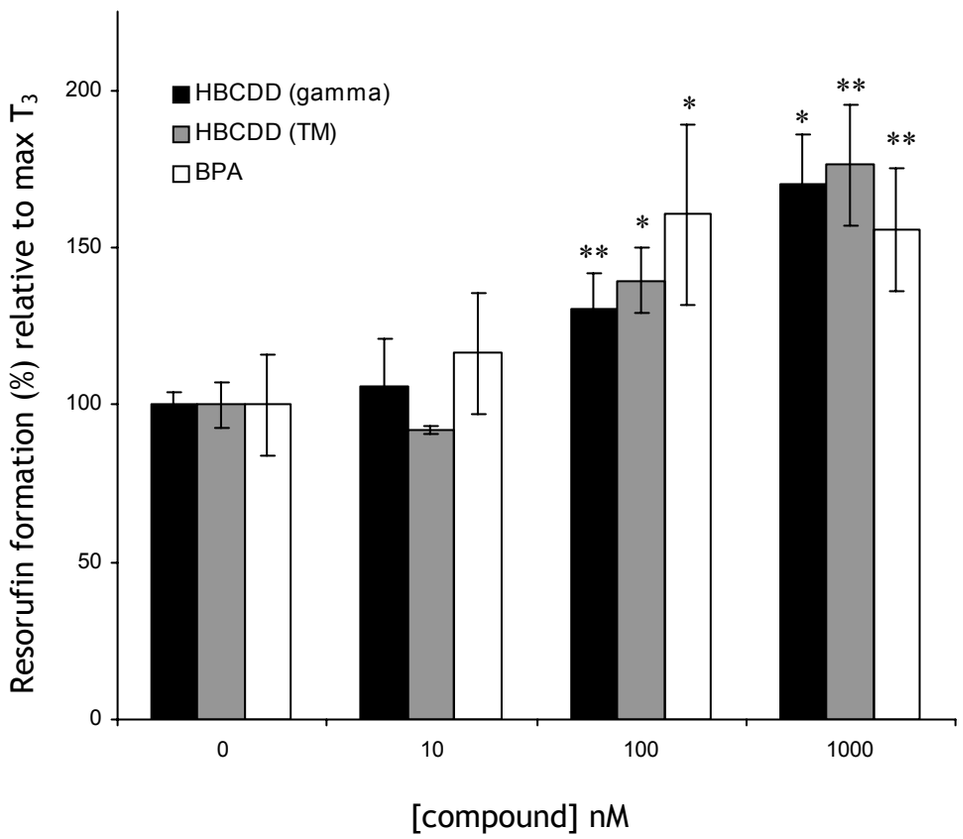


Figure 7. Effect of different concentrations of HBCD-γ, technical mixture (TM) and bisphenol A (BPA) on rat GH_3 cell proliferation in presence of 0.25 nM T_3. Cells were plated (2500 cells/well) and exposed to compounds in serum free PCM medium for 96 h. Error bars were calculated as SD and data presented are means from triplicate data points and expressed as percentage to the blanc which was set at 100 %. Absolute numbers for control values were: 4315 (solvent control) – 8791 (1000 nM T_3) RFU. * Significantly different from control (p < 0.05); ** p < 0.01

Discussion

The present study describes optimization and validation of the T-screen assay providing the possibility to investigate the involvement of TR mediated

mechanisms in the cellular effects evoked by thyroid hormone disruptors. To this end the specificity of the T-screen to detect xenobiotics with known agonistic and antagonistic activity was tested and the responses quantified. The T-screen was further optimized by addition of fetuin, a serum glycoprotein, to the serum free PCM medium. Fetuin overcomes poor attachment and spreading of the GH_3 cells in serum-free medium (Brunet et al., 1982), and greatly promotes cell proliferation, thereby enhancing the resolution of the assay. To be able to distinguish pure cell proliferation from increased mitochondrial activity (possibly induced by T_3-like compounds) without increased cell proliferation, the response in the resazurine-assay was compared with the response of the BrdU-assay only indicating DNA replication. The results demonstrate that in our studies the resazurine assay does reflect GH_3 cell proliferation. However, caution should be taken when testing reducing compounds, because they may cause resazurine reduction that does not reflect cell proliferation. This can be confirmed by performing the assay with the highest concentration of the test compound in the absence of cells as well. After optimization of the T-screen the agonistic action of T_2-, T_3- and T_4-like model hydroxylated BDEs was compared. As expected the T_3-like BDE-OH was the most potent agonist in the T-screen followed by the T_4-like BDE-OH with a roughly six-fold lower EC_{50} value, whereas non-hydroxylated BDEs do hardly or not induce TR-mediated responses in the T-screen. The three model hydroxylated BDEs were not selected because they are relevant *in vivo*, but were synthesized as model compounds with potential high affinity for the TR (Marsh et al., 1998). The relative potencies of these hydroxylated BDEs are in good agreement with their activity in the binding assay with isolated TRs (Marsh et al., 1998). The EC_{50}'s in the TR binding assay are roughly 6 fold lower than the EC_{50}'s observed in the T-screen. This is not surprising as in the T-screen compounds have to pass a cell membrane, which is absent in studies with isolated receptors. Other xenobiotics were tested, including a series of brominated diphenylethers (BDEs), known to displace T_4 from the human TTR (Meerts et al., 2000). Our results show that most of these xenobiotics are not active in the T-

screen. This is an important finding because it illustrates that non-hydroxylated BDEs are more active in displacing T_4 from TTR than in TR mediated cellular responses, and provides mechanistic insight in the level at which these compounds are likely to disturb the thyroid hormone system. As earlier suggested (Dietrich et al., 1977) it appears that the presence of a 4'-hydroxyl moiety and a 3'-substituent are prerequisites for TR binding affinity. Since during *in vivo* metabolization formation of T-screen active 4'-hydroxylated BDEs may take place. As GH_3 cells do not possess cytochrome P450 activity (Schriks et al., unpublished results.), some parent BDEs (BDE69 and BDE75) were metabolized with rat hepatic P450 2B enzymes after which the incubation extracts were tested in the T-screen. None of the extracts were able to induce GH_3 cell proliferation (data not shown), but it cannot be excluded that this may be related to the actual level of conversion achieved in the microsomal incubations. Even when 5 % could be converted in the right hydroxylated metabolites as was shown in previous experiments with the highly metabolizable 3,4,3',4'-tetrachlorobiphenyl (Murk et al., 1994), this would probably not allow T-screen induction before the still present parent compound would become cytotoxic to the GH_3-cells. *In vivo*, however, there is selective retention of hydroxylated metabolites in mammalian blood resulting in levels that are even higher than those of the parent compound (Klasson-Wehler et al., 1989). Therefore, further testing should include isolation of hydroxylated metabolites after metabolic conversion of BDEs to allow conclusions about the thyromimicking potency of other 4'-hydroxylated metabolites as suggested (Marsh et al., 1998). A potentiation was clearly revealed for BPA, HBCD (TM) and HBCD-γ in combination with T_3, which did not respond in the T-screen alone. This is a very relevant finding as T_3 and T_4 always are present *in vivo* (T_3: 13.9 nM – 26.4 nM; T_4: 80 nM – 103 nM) (Henneman, 1986). Although HBCD has been detected in serum of Dutch mothers at much lower concentrations (1.25 pM – 54.6 pM) (Weis et al., 2004), than the concentrations that were potentiating in the T-screen, they could contribute to possible potentiating effects in mixtures with other potentiating compounds. The mechanism underlying these

potentiating effects are still unknown, but we are currently studying possible interference with regulation of TR-mediated gene transcription. TBBPA and TCBPA did not induce T-screen response up to cytotoxic concentrations > 1 μM. This is in contradiction with earlier results reported by Kitamura and co-workers, who observed potent thyromimicking effects at 100 μM of these compounds (absence of T_3) in a comparable GH_3 cell line (Kitamura et al., 2002). The reasons for this discrepancy in cytotoxicity are unclear, but might be explained by usage of a different GH_3 cell clone. Interestingly, Kitamura and co-workers report anti-thyroid hormone action of TBBPA and TCBPA in a thyroid hormone-responsive reporter assay using a Chinese hamster ovary cell line (CHO-K1) transfected with TRα or TRβ. The authors subsequently hypothesize that TBBPA and TCBPA may show a different mode of action in different cell lines (Kitamura et al., 2005). Another explanation for the observed discrepancy could be usage of different batches of TBBPA and TCBPA. To eliminate the possibility of DMSO interfering with the observed effects, a different solvent was tested but results obtained with ethanol instead of DMSO as the solvent were similar (data not shown). The specificity of the T-screen for antagonistic compounds was demonstrated by the IC_{50} (2.1 μM) for the known and specific antagonist amiodarone (Goldfine et al., 1982). The potency ratio of amiodarone relative to T_3 is in the same order of magnitude as earlier reported (amiodarone 3.3 ± 0.2 μM; T_3 0.87 ± 0.2 nM) (Norman et al., 1989). BDE206 was the only, but very potent, TR-antagonist in our study. This antagonistic potency (IC_{50} = 449.2 nM) was even five times stronger than that of the positive control amiodarone. The exact mechanism underlying the observed antagonistic action of BDE206 still is unknown, but to our knowledge this reporting is the first time that a higher brominated diphenylether consistently elicits anti-thyroid hormone action. This is an important finding, as higher brominated diphenylethers are used extensively at the present in for example plastics, textiles, electronic circuitry and many other materials to prevent fires, and BDE206 has already been detected at relatively high concentrations in sediment (14.2 nmole.kg^{-1} dry wt.) (Guardia et al., 2004). Furthermore, increasing

evidence demonstrates that higher brominated diphenylethers can be debrominated due to photo-decomposition to lower brominated forms. (Guardia et al., 2004; Soderstrom et al., 2004; Watanabe et al., 1987). In conclusion, the results of the present study show that the T-screen can be used as a fast screening tool for identification of compounds that can induce functional agonistic, antagonistic and potentiating TR-mediated effects and to quantify this thyroid hormone disrupting effect.

Acknowledgements

The authors would like to thank prof. Åke Bergman of Stockholm University for the provision of the synthesized hydroxylated BDEs, and dr. Timo Hamers (Institute for Environmental Studies, The Netherlands) for preparation of compound stock solutions. This research was financially supported by a CEFIC-EMSG grant (project coordinator Christ de Rooij).

References

Besselink, H. T., Brouwer, A. and Vethaak, A. D., 1996. Effect on retinoid homeostasis in the flounder (*Platichthys flesus*) following chronic exposure to contaminated harbour dredge spoil from Rotterdam harbour. **Organohalogen Compounds 29**, 18-21.

Brouwer, A., Berg, K. J. v. d., Blaner, W. S. and Goodman, D. S., 1986. Transthyretin (prealbumin) binding of PCBs, a model for the mechanism of interference with vitamin A and thyroid hormone metabolism. **Chemosphere 15**, 1699-1706.

Brouwer, A., Klasson-Wehler, E., Bokdam, M., Morse, D. C. and Traag, W. A., 1990. Competitive inhibition of thyroxin binding to transthyretin by monohydroxy metabolites of 3,4,3',4'-tetrachlorobiphenyl. **Chemosphere 20**, 1257-1262.

Brunet, N., Gourdji, D., Tixier-Vidal, A., Rizzino, A., 1982. Growth of cells in hormonally defined media. Cold Spring Harbour Laboratory, New York.

Colborn, T., 2002. Clues from wildlife to create an assay for thyroid system disruption. **Environ Health Persp 110** Suppl 3, 363-367.

Crofton, K. M., 2004. Developmental disruption of thyroid hormone: Correlations with hearing dysfunction in rats. **Risk Anal 24**, 1665-1671.

Darnerud, P. O., Morse, D., Klasson-Wehler, E. and Brouwer, A., 1996. Binding of a 3,3',4,4'-tetrachlorobiphenyl (CB-77) metabolite to fetal transthyretin and effects on fetal thyroid hormone levels in mice. **Toxicology 106**, 105-114.

Dietrich, S. W., Bolger, M. B., Kollman, P. A. and Jorgensen, E. C., 1977. Thyroxine analogues. 23. Quantitative structure-activity correlation studies of *in vivo* and *in vitro* thyromimetic activities. **J Med Chem 20**, 863-880.

Fisher, H. W., Puck, T. T. and Sato, G., 1958. Molecular growth requirements of single mammalian cells: The action of fetuin in promoting cell attachment to glass. **Proc Natl Acad Sci USA 44**, 4-10.

Gingrich, S. A., Smith, P. J., Shapiro, L. E. and Surks, M. I., 1985. 5,5'-Diphenylhydantoin (phenytoin) attenuates the action of 3,5,3'- triiodo-L-thyronine in cultured GC cells. **Endocrinology 116**, 2306-2313.

Goldfine, I. D., Maddux, B., Woeber, K. A., 1982. Effect of amiodarone on 3,3',5-triiodo-L-thyronine stimulation of [^{3}H] thymidine incorporation into GH_3 cells. **J Endocrinol Invest 5**, 165-168.

Guardia, L. M. J., Hale, R. C. and Harvey, E., 2004. Environmental debromination of decabrominated diphenyl ether. Proc. The Third International Workshop on Brominated Flame Retardants (BFR2004), University of Toronto, Toronto, Ontario, Canada.

Gutleb, A. C., Appelman, J., Bronkhorst, M., van den Berg, J. H. J. and Murk, A. J., 2000. Effects of oral exposure to polychlorinated biphenyls (PCBs) on the development and metamorphosis of two amphibian species (*Xenopus laevis* and *Rana temporaria*). **Sci Total Environ 262**, 147-157.

Gutleb, A. C., Meerts, I. A. T. M., Bergsma, J. H., Schriks, M. and Murk, A. J., 2005. T-Screen as a tool to identify thyroid hormone receptor active compounds. **Environ Toxicol Phar 19,** 231-238.

Henneman, G., 1986. Thyroid hormone metabolism. Marcel Dekker, Inc., New York.

Hinkle, P. M. and Kinsella, P. A., 1986. Thyroid hormone induction of an autocrine growth factor secreted by pituitary tumor cells. **Science 234**, 1549-1552.

Jung, R. E. and Walker, M. K., 1997. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on development of anuran amphibians. **Environ Toxicol Chem 16**, 230-240.

Kitamura, S., Jinno, N., Ohta, S., Kuroki, H. and Fujimoto, N., 2002. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. **Biochem Biophys Res Commun 293**, 554-559.

Kitamura, S., Kato, T., Iida, M., Jinno, N., Suzuki, T., Ohta, S., Fujimoto, N., Hanada, H., Kashiwagi, K. and Kashiwagi, A., 2005. Anti-thyroid hormonal activity of tetrabromobisphenol A, a flame retardant, and related compounds: Affinity to the mammalian thyroid hormone receptor, and effect on tadpole metamorphosis. **Life Sci 76**, 1589-1601.

Lans, M. C., Klasson-Wehler, E., Willemsen, M., Meussen, E., Safe, S. and Brouwer, A., 1993. Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-p-dioxins and -dibenzofurans with human transthyretin. **Chem Biol Interactions 88**, 7-21.

Lans, M. C., Spiertz, C., Brouwer, A. and Koeman, J. H., 1994. Different competition of thyroxine binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs and PCDFs. **Eur J Pharmacol 270**, 129-136.

Legrand, J., 1986. Thyroid hormone effects on growth and development. Thyroid Hormone Metabolism. Hennemann G. 627, 503-534.

Leung, H. W., Paustenbach, D. J., Murray, F. J. and Andersen, M. E., 1990. A physiological pharmacokinetic description of the tissue distribution and enzyme-inducing properties of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the rat. **Toxicol Appl Pharm 103**, 399-410.

Mann, D. N. and Surks, M. I., 1983. 5,5'-Diphenylhydantoin decreases specific 3,5,3'-triiodothyronine (T_3) binding by rat hepatic nuclear T_3 receptors. **Endocrinology 112**, 1723-1731.

Marsh, G., Bergman, A., Bladh, L.-G., Gillner, M. and Jackobsson, E., 1998. Synthesis of p-Hydroxybromodiphenyl Ethers and binding to the thyroid receptor. **Organohalogen Compounds 37**, 305-309.

Meerts, I. A., van Zanden, J. J., Luijks, E. A., van Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A. and Brouwer, A., 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. **Toxicol Sci 56**, 95-104.

Murk, A., Morse, D., Boon, J. and Brouwer, A., 1994. *In vitro* metabolism of 3,3',4,4'-tetrachlorobiphenyl in relation to ethoxyresorufin-O-deethylase activity in liver microsomes of some wildlife species and rat. **Eur J Pharmacol 270**, 253-261.

Murk, A. J., Vandenberg, J. H. J., Fellinger, M., Rozemeijer, M. J. C., Swennen, C., Duiven, P., Boon, J. P., Brouwer, A. and Koeman, J. H., 1994. Toxic and Biochemical Effects of 3,3',4,4'-Tetrachlorobiphenyl (Cb-77) and Clophen A50 on Eider Ducklings (Somateria- Mollissima) in a Semifield Experiment. **Environ Pollut 86**, 21-30.

Murk, A. J., Leonards, P. E. G., van Hattum, B., Luit, R., van der Weiden, M. E. J. and Smit, M., 1998. Application of biomarkers for exposure and effect of polyhalogenated aromatic hydrocarbons in naturally exposed European otters (*Lutra lutra*). **Environ Toxicol Pharmacol 6**, 91-102.

Norman, M. F. and Lavin, T. N., 1989. Antagonism of thyroid hormone action by amiodarone in rat pituitary tumor cells. **J Clin Invest 83,** 306-13.

O'Brien, J., Wilson, I., Orton, T. and Pognan, F., 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. **Eur J Biochem / FEBS 267**, 5421-5426.

Sirbasku, D. A., Pakala, R., Sato, H. and Eby, J. E., 1991. Thyroid hormone dependent pituitary tumor cell growth in serum-free chemically defined culture. A new regulatory role for apotransferrin. **Biochemistry 30**, 7466-7477.

Smith, P. J. and Surks, M. I., 1984. Multiple effects of 5,5'-diphenylhydantoin on the thyroid hormone system. **Endocr Rev 5**, 514-524.

Snodgrass, J. W., Hopkins, W. A., Broughton, J., Gwinn, D., Baionno, J. A. and Burger, J., 2004. Species-specific responses of developing anurans to coal combustion wastes. **Aquat Toxicol 66**, 171-182.

Soderstrom, G., Sellstrom, U., de Wit, C. A. and Tysklind, M., 2004. Photolytic debromination of decabromodiphenyl ether (BDE 209). **Environ Sci Technol 38**, 127-132.

Veldhoen, N. and Helbing, C. C., 2001. Detection of environmental endocrine-disruptor effects on gene expression in live *Rana catesbeiana* tadpoles using a tail fin biopsy technique. **Environ Toxicol Chem 20**, 2704-2708.

Watanabe, I. and Tatsukawa, R., 1987. Formation of brominated dibenzofurans from the photolysis of flame retardant decabromobiphenyl ether in hexane solution by UV and sun light. **Bull Environ Contam Toxicol 39**, 953-959.

Weis, J. S., Meijer, L., Sauer, P., Linderholm, L., Athanassiadis, I. and Bergman, A., 2004. PBDE and HBCDD levels in blood from Dutch mothers and infants - Analysis of a Dutch Groningen infant cohort. **Organohalogen Compounds 66**.

Zemel, L. R., Biezunski, D. R., Shapiro, L. E. and Surks, M. I., 1988. 5,5'-Diphenylhydantoin decreases the entry of 3,5,3'-triiodo-L- thyronine but not L-thyroxine in cultured GH-producing cells. **Acta Endocrinol (Copenh) 117**, 392-398.

3

Thyroid hormone receptor isoform selectivity of thyroid hormone disrupting compounds quantified with an *in vitro* reporter gene assay

Merijn Schriks[1], Julie M. Roessig[2], Albertinka J. Murk[1], J. David Furlow[2]

[1]Division of Toxicology, Wageningen University, Wageningen, The Netherlands
[2]Dept. of Neurobiology, Physiology and Behavior, University of California, Davis, USA

Submitted to Environmental Toxicology and Pharmacology

Abstract

Some compounds, including brominated diphenylethers (BDEs), can interfere with Thyroid hormone (TH) Receptor (TR)-mediated TH-signalling. In this study, the TR isoform selectivity of some TH disrupting compounds was investigated with TRα/β specific reporter gene assays. For this purpose, the effects of compounds on 3,3',5-triiodothyronine (T_3)-induced TRα- or TRβ-activation were tested in green monkey kidney fibroblast (CV-1) cells transiently transfected with *Xenopus laevis* TRs and a luciferase reporter gene. The T_3-like BDE-OH and diiodobiphenyl (DIB) increased T_3-induced TRα-activation, but not T_3-induced TRβ-activation. BDE28 (100 nM) did not act via TRα, but almost tripled T_3-induced TRβ-activation relative to T_3 at its EC_{50}. BDE206 (100 nM) was antagonistic on both TRs with a maximum repression -54 % relative to T_3 at its EC_{50}. Contrary to previous results obtained with the T-screen, HBCD was inactive. The present study illustrates the importance of testing potential TH disrupting compounds in model systems that enable independent characterization of effects on both T_3-induced TRs.

Introduction

Thyroid hormones (THs) regulate several essential physiological processes such as energy metabolism, growth, formation of the central nervous system (CNS), tissue differentiation and reproduction (Tata, 1993; Kaltenbach, 1996; Wu and Koenig, 2000). The molecular action of THs is mediated via the Thyroid hormone Receptors (TRs) (Wu and Koenig, 2000) which, after ligand binding, activate genes by binding to the thyroid hormone response elements (TREs). Vertebrates have two separate genes that encode the TRs, designated TRα and TRβ (Sap et al., 1986; Weinberger et al., 1986).

Considering the importance of THs on various physiological and morphogenetical processes, it is not surprising that the interference of environmental contaminants on multiple levels of thyroid hormone signalling is of concern to both scientists as well as to legislators demanding that newly marketed compounds are tested for TH disrupting potential (Devito et al., 1999; OECD, 2006). A group of persistent organic pollutants (POPs) known to interfere with the TH-axis at several levels, are the polyhalogenated aromatic hydrocarbons (PHAHs). These compounds and their hydroxylated metabolites can compete with 3,3′,5,5′-L-thyroxine (T_4) for binding to the plasma transport protein transthyretin (TTR) (Lans et al., 1993, 1994; Cheek, 1999; Meerts et al., 2000; Ishihara et al., 2003; Yamauchi et al., 2000, 2003) and they also inhibit deiodinase activity (Adams et al., 1990), T_4-glucuronidation and T_4-sulfation (Schuur et al., 1998ab; Brouwer et al., 1998). Only recently, more attention is paid to direct interaction with the TRs and related complex effects on TH signalling (Zoeller, 2005). In recent TR-binding studies it has been shown that bisphenol-A binds to TRα/β with relative high affinity (Kitamura et al., 2002; Moriyama et al., 2002), but that polychlorinated biphenyls (PCBs) do not (Gauger et al., 2004). In the presence of the most bioactive form of TH, 3,3′,5-triiodo-L-thyronine (T_3), it is to be expected that specific compounds can bind to and/or modulate the activity of TRα or TRβ in a highly selective way (McKinney and Waller, 1994, 1998). Beeren (1995) and Bakker (1994) showed, for example, that the pharmaceutical

compound desethylamiodarone is a noncompetitive inhibitor of 3,3',5-triiodo-L-thyronine (T_3) binding to TRβ, but a competitive inhibitor of T_3 binding to TRα.

The aim of the present study was to obtain more insight in the possible TR-isoform selectivity of thyroid hormone disrupting compounds with the aid of TRα/β specific reporter gene assays. Since T_3 is present under physiological conditions *in vivo*, the selected compounds were tested in combination with T_3 at its EC_{50}. This concentration is representative for T_3-concentrations in the second trimester of human fetal development, during which vulnerable important morphogenetical processes take place that give rise to the final structure of the CNS, including the brain (Zoeller et al 2004).

To reach the aim as stated earlier, the green monkey kidney fibroblast (CV-1) cell line was transfected with either Xenopus TRα (xTRα) or Xenopus TRβ (xTRβ) and a BTEB TRE ΔMTV luciferase reporter plasmid (Furlow et al., 2004). CV-1 cells were used for this study since they have been applied widely to examine regulation of gene expression by T_3 and do not express endogenous TRs (Yen et al., 1995; Laflamme et al., 2002). The compounds tested are brominated diphenylethers (BDEs) and other halogenated hydrocarbons that have previously been shown to be active in the T-screen, an *in vitro* T_3-dependent proliferation assay (Schriks et al., 2006). They are either agonizing (T_3-like BDE-OH), antagonizing (BDE206) or potentiating (diiodobiphenyl, BDE28 and hexabromocyclododecane) T_3-action in the TR-mediated T-screen.

Materials and Methods

Compounds

The brominated diphenylethers BDE28 [bromine substitution pattern 2,4,4'], BDE206 [2,2',3,3',4,4',5,5',6] (>99 %) and 4'-hydroxy-2,4,6-tribromodiphenyl ether (further abbreviated as T_3-like BDE-OH) (>99 %) were kindly provided by prof. dr. Åke Bergman (Stockholm University, Sweden) and also tested within the framework of the EU-funded FIRE project. Hexabromocyclododecane (HBCD, technical mixture (TM); no data on impurities supplied by the provider) was

obtained through BSEF (Brussels, Belgium), and 4,4'-diiodobiphenyl (DIB) (>98%) from ABCR GmbH & Co (Karlsruhe, Germany). All compounds were dissolved in dimethylsulfoxide (DMSO, 99.9 %; Acros Organics, Geel, Belgium) and kept at room temperature in the dark, except T_3-stocks which were stored at –80 °C and the T_3-like BDE-OH which was stored at -18 °C. Figure 1 presents the chemical structure of the model compounds used in this study. All other chemicals were purchased from Sigma unless otherwise stated.

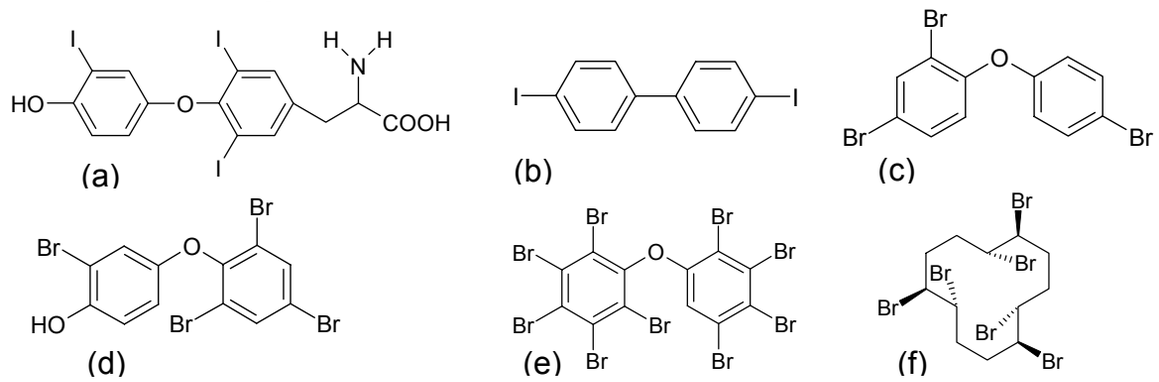


Figure 1. Chemical structures of (a) 3,3',5-triiodo-L-thyronine (T_3); (b) 4,4'-diiodo biphenyl (DIB); (c) 2,4,4'-brominated diphenylether (BDE28); (d) 4'-hydroxy-2,4,6-brominated diphenylether (T_3-like BDE-OH); (e) 2,2',3,3',4,4',5,5',6-brominated diphenylether (BDE206); (f) hexabromo cyclododecane (TM) (HBCD).

Transient transfection assays

The green monkey kidney fibroblast (CV-1) cell line (cell culture facility UC Davis) was grown to ~50 % confluency in Dulbecco's Modified Eagle's Medium (DMEM) containing glucose, L-glutamine, pyridoxine hydrochloride, 110 mg.L^{-1} sodium pyruvate, 1 % antibiotic–antimycotic solution (Gibco Invitrogen Corporation), and 5 % heat-inactivated fetal bovine serum (FBS). Transient transfections were performed as described (Furlow et al., 2004) with modifications. Briefly, CV-1 cells (~2 x 10^4 cells/well) were transfected with 50 ng X. laevis TR expression vector (miw xTRαA or miw xTRβA1), 100 ng BTEB TRE ΔMTV luciferase reporter plasmid, and 100 ng pCS2-β-galactosidase (Furlow et al., 2004). Prior to transfections, DNA was pre-mixed in polystyrene tubes with 2 µL Lipofectamine™2000 (Invitrogen) per well in Minimal Essential Medium

(MEM) and added to cells for 6 h. Cells were allowed to recover overnight in DMEM supplemented with 5 % AG-X8 resin stripped FBS (Samuels et al., 1979). Exposure was during 48 h to T_3 (0.1-1000 nM in 10-fold dilution steps), solvent-controls and compounds in combination with T_3 at its EC_{50} (0.5 nM and 1.5 nM T_3 for TRα and TRβ, respectively). DMSO concentrations in the wells never exceeded 0.5 % (v/v). The model compounds were tested in a concentration range that had been shown before not to be cytotoxic. Cells were harvested by scraping in buffer (10 mM Tris-HCl, 500 mM NaCl, and 1 mM EDTA, pH 7.6), spinning down (14.000 rpm) and re-suspension of cell pellets in 100 µL reporter lysis buffer (Promega). Cell lysates were assayed for luciferase and β-galactosidase activity as described elsewhere (Brasier et al., 1989; Sambrook et al., 1989).

Statistical analysis

The model compounds were all tested in combination with the EC_{50} of T_3 and the effect generated by T_3 at its EC_{50} was set at 100 %. Statistical analysis of the data was carried out using the Student's *t*-test. The acceptance level was set at $p \leq 0.05$.

Results

Activation of TRs by T_3

T_3 induced luciferase production in transiently transfected CV-1 cells for both TR isoforms in a dose-dependent fashion (Figure 2). Maximal TR-activation for both isoforms occurred at 100 nM T_3 and luciferase induction was 74 ± 8 and 45 ± 6 fold for TRα and TRβ, respectively. At higher concentrations T_3 the responses decreased without visible signs of cytotoxicity. The EC_{50} for TRα- and TRβ-activation by T_3 were 0.5 nM and 1.5 nM, respectively. These concentrations were used for further experiments in combination with the tested compounds.

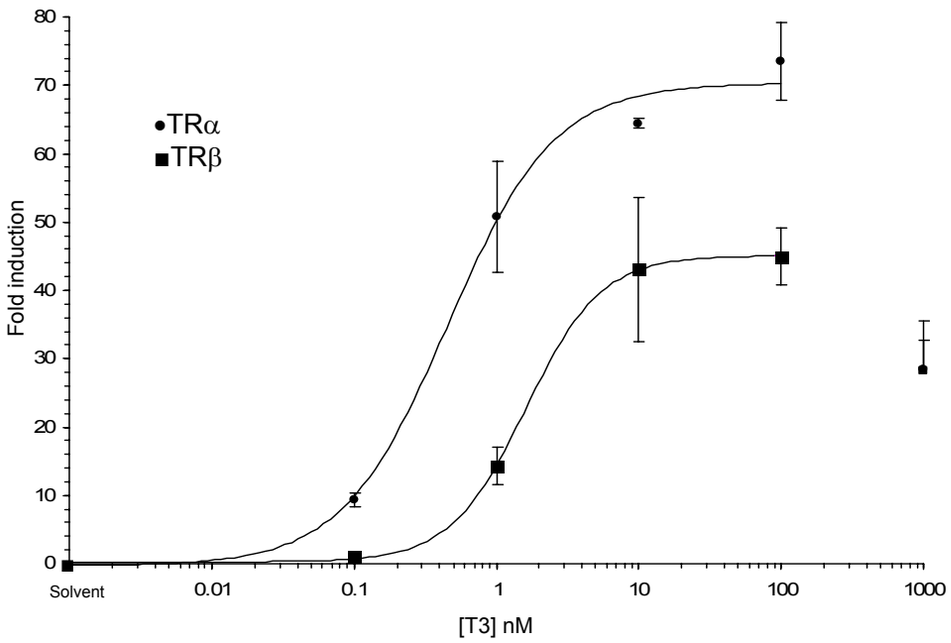


Figure 2. T_3-induced luciferase induction in thyroid hormone (TR)-specific transient reporter gene assays. A thyroid hormone response element (TRE)-containing promotor driving luciferase reporter gene expression was co-transfected into green monkey kidney cells (CV-1) along with expression vectors for *Xenopus laevis* TRα (circles) or TRβ (TRβ; squares). Luciferase activity was normalized to constitutive β-galactosidase activity. Each data point represents the average ± the standard deviation of triplicate measurements.

Effects of compounds on T_3 (EC_{50})-induced activation of TRs

The T_3-like BDE-OH, which was also used as an agonist in the T-screen, increased T_3-induced TRα-activation (Figure 3a). At 1 μM this effect was +73 % relative to the effect observed with T_3 at its EC_{50}. BDE206 significantly antagonized the effect of T_3 at its EC_{50} via both TRs already at a concentration of 100 nM (Figure 3b). The effect of 1 μM BDE206 on the T_3-induced TRβ-activation was -54 %, whereas the effect on the T_3-induced TRα-activation was -30 %.

Of the three model potentiating compounds tested, DIB increased T_3-induced TRα-activation with an additional +36 % relative to the effect of T_3 at its EC_{50} (Figure 3c). The most potent compound tested was BDE28, which almost tripled the T_3-induced TRβ-activation (+274 % at 100 nM) but did not affect T_3-induced TRα-activation at all (Figure 3d). Of the model compounds selected, only HBCD

did not show any TRα- nor TRβ-activation (Figure 3e), despite the fact that HBCD potentiated T_3-action in the T-screen (Schriks et al., 2006).

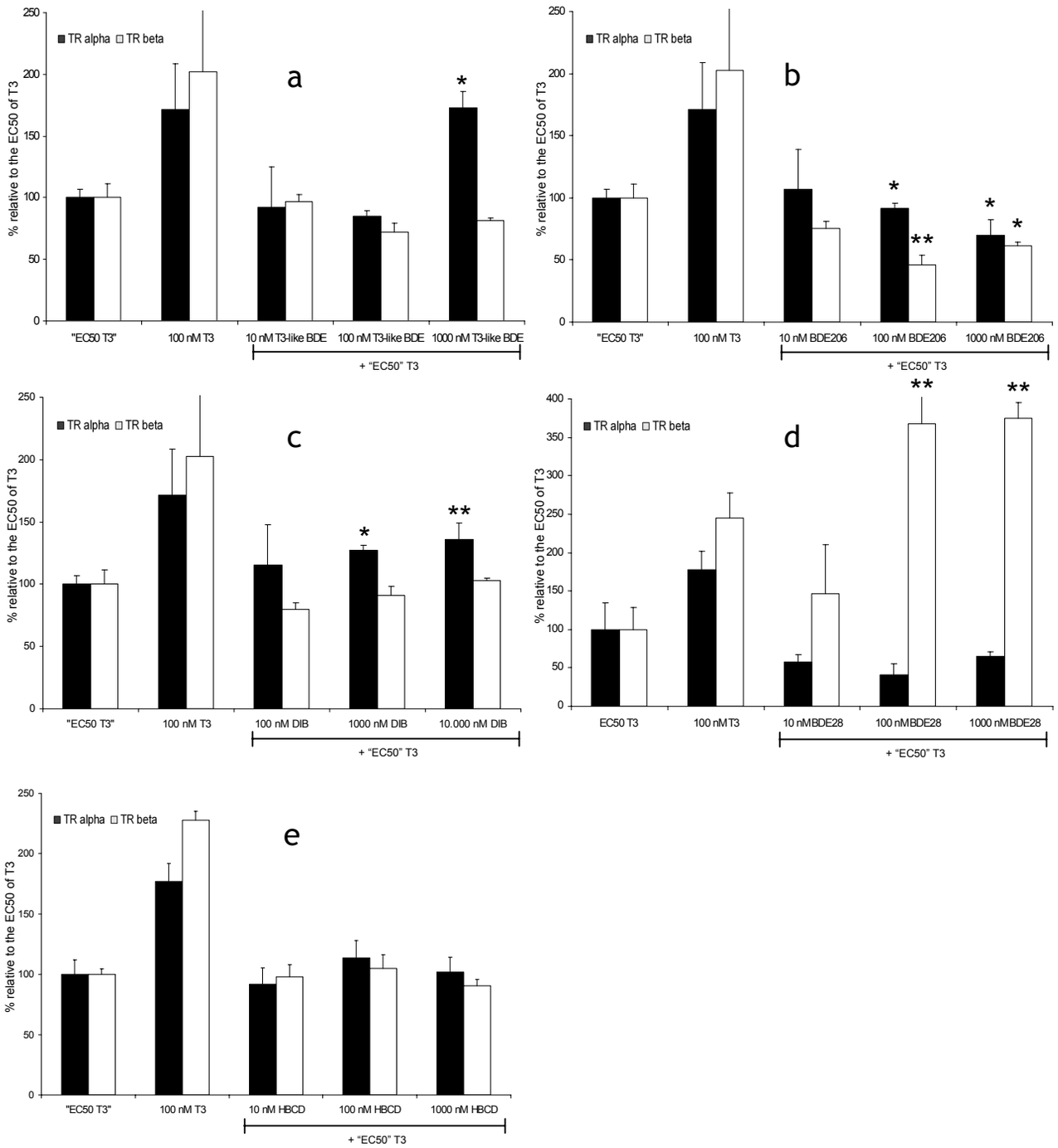


Figure 3. Effects of model compounds on $T_3(EC_{50})$-induced TRα- and TRβ-activation in transiently transfected CV-1-luc cells: (a) T_3-like BDE-OH; (b) BDE206; (c) DIB; (d) BDE28; (e) HBCD. Luciferase activity was normalized to constitutive β-galactosidase activity. The T_3-response at the EC_{50} was set at 100 %. Each data point represents the average ± the standard deviation of triplicate measurements. * Significantly different from T_3 at its EC_{50}-value ($p \leq 0.05$); ** $p \leq 0.01$.

Discussion

The aim of the present study was to investigate T_3-induced thyroid hormone receptor (TR) isoform selectivity of a panel thyroid hormone disrupting compounds. The results obtained reveal differences in T_3-induced TR isoform selectivity of compounds as measured with a CV-1 cell line transiently transfected with either TRα or TRβ. The EC_{50} for TRα-activation by T_3 was roughly 3 times lower than the EC_{50} for TRβ-activation. T_3 tested in a radio-ligand displacement assay with purified human TRα or TRβ showed a comparable difference in binding affinity (Chiellini et al., 1998). Considering the importance of T_3 for the *in vivo* situation, the model compounds were all tested in the presence of T_3 at its EC_{50} and the effect induced by T_3 at its EC_{50} was set at 100 %. Table 1 summarizes the results for the tested compounds in the transient TRα and TRβ reporter gene assay and also presents the results obtained for these compounds in the T-screen assay to allow easy comparison of their responses in the present study to those in the T-screen.

Table 1. Effects of compounds on T_3-induced TRα- or TRβ-activation in a transient reporter gene assay expressed relative to the EC_{50} of T_3 which was set at 100 % (Avg ± Std) (concentration bracketed). The abbreviations and structures of the model compounds are presented in Figure 1. The basis of the selection of the model compounds, the effects in the T_3-dependent proliferation assay (T-screen), are presented in italics.

	T-screen	*T-screen*	TRα	TRβ	LOEC TRα / TRβ
EC_{50} T_3 (nM)		*0.21 ± 0.12*[d]	0.5	1.5	0.1 / 1
Compounds	*Class*	Max. induction (+) or repression(-) relative to EC_{50}			
T_3-like BDE-OH	*Agonist*	*+97 ± 6 (1 μM)*[d]	+73 ± 17 (1 μM)[a]	-[c]	1 / -
BDE206	*Antagonist*	*-58 ± 6 (10 μM)*[d]	-30 ± 5 (1 μM)[a]	-54 ± 8 (0.1 μM)[b]	0.1 / 0.1
DIB	*Potentiator*	*+32 ± 2 (10 μM)*[e]	+36 ± 1 (10 μM)[b]	-[c]	1 / -
BDE28	*Potentiator*	*+66 ± 3 (1 μM)*[f]	-[c]	+274 ± 15 (0.1 μM)[b]	- / 0.1
HBCD	*Potentiator*	*+76 ± 10 (1 μM)*[d]	-[c]	-[c]	>1 / >1

LOEC is the lowest observed effect concentration in either the TRα or the TRβ
[a] $p \leq 0.05$; [b] $p \leq 0.01$; [c] No significant effect; [d] Schriks *et al.*, 2006; [e] Schriks *et al.*, unpublished data; [f] Hamers *et al.*, 2006.

The T_3-like BDE-OH selectively increased T_3-mediated TRα-activation only (Table 1), which may be in contrast with previous human TR binding affinity studies carried out by Marsh and co-workers (1998) who report preferential TRβ-binding

over TRα-binding. However, the outcome of both studies may not be directly comparable since the present study encompasses the complete cellular signal transduction cascade involved in TR-activation, instead of TR binding only. BDE206 tested in the presence of T_3 at its EC_{50}, was antagonistic via both TRs in the same concentration range and to the same degree (for TRβ) as previously reported for the T-screen (Table 1) (Schriks et al., 2006). These results are in also accordance with the antagonistic effects of BDE206 on *in vivo* cell proliferation in the rostral head region of one-week old *Xenopus laevis* tadpoles (Schriks et al., subm. 2006a) and on T_3-induced regression of isolated tail tips from premetamorphic *Xenopus laevis* tadpoles (Schriks et al ., 2006b).

Of the three potentiating compounds selected based on the T-screen results, DIB and BDE28 increased the TR-mediated T_3-response, but via different TRs. The degree of TRα-mediated stimulation of the T_3-response by DIB is in accordance with the effect in the T-screen (Schriks et al., 2006). In both cases the maximum additional effect is roughly 35 % relative to the EC_{50} response of T_3, and the maximum T_3-response is not yet reached at 10 μM of DIB. The stimulation of T_3-induced TRα-activation by DIB also is in accordance with the effects previously shown in HeLaTR reporter cells endogenously expressing the human TRα (Yamada-Okabe et al., 2005). The same authors also report estrogen receptor (ER)-α mediated gene expression, suggesting that DIB may also induce the response via the estrogen-route. In the present study, however, cross reactivity with the ERs can be excluded since CV-1 cells are devoid of endogenous ERs (Yen et al., 1995).

BDE28 specifically increased T_3-induced TRβ- but not TRα-activation in the transiently transfected CV-1 cells. At 100 nM BDE28 the total response was even 50 % higher than the maximum induction by 100 nM T_3. BDE28 also potentiated the T_3-action in the T-screen, but only with an additional 66 % to the EC_{50} effect of T_3, and the maximal response was not yet reached at 1 μM (Table 1) (Hamers et al., 2006). Compounds that increase T_3-induced TR-activation may be stimulating cofactor renewal and/or recruitment, but other explanations are possible as well

and the exact mechanism remains to be investigated. As CV-1 cells lack cytochrome P4501A1 activity (Hoffer et al., 1996), the formation of highly reactive metabolites is unlikely.

In contrary to DIB and BDE28, HBCD did not affect T_3-induced TRα- or TRβ-activation in the present study. However, in combination studies with HeLa- and GH_3-cells (Yamada-Okabe et al., 2005; Schriks et al., 2006) and *Xenopus laevis* tissues (Schriks et al., 2006b) HBCD consistently increased the T_3-response. An important difference between these *in vitro*/*ex vivo* models and CV-1 cells, is that the latter cell line does not contain endogenous TRs, and therefore possibly also lack related specific sites that can be targeted by HBCD. For example, HBCD may affect cell proliferation of GH_3 cells in the T-screen by influencing cell-specific cell cycle regulators such as cyclin or cyclin-dependent kinase levels and activity (Barrera-Hernandez et al., 1999). This suggests that potentiation of T_3-action observed earlier for HBCD, occurs via a different mechanism than for DIB and BDE28 which more closely resemble the molecular structure of T_3 (Figure 1). It is very relevant to further elucidate the mechanistic action of HBCD, which could have implications for risk assessment. This was, however, beyond the scope of the present study.

All together, the effects of compounds on TRα/β-specific activation as observed may imply that differential physiological effects on TRα or TRβ dominated tissues can occur after exposure to compounds. Such tissue specific effects are demonstrated by Furlow and co-workers (2004) who show that treatment of *Xenopus laevis* tadpoles with the pharmaceutical TRβ agonist GC-1 only induces precocious resorption of TRβ dominated tissues such as gills and the tail. GC-1 has limited effect on tissues such as hind limbs which express high TRα levels. Likewise, the compounds tested in this study may exert similar responses under physiological conditions in humans and other vertebrates. For example the vertebrate CNS and brain exhibit distinct temporal and spatial patterns of TRα and TRβ expression (Bradley et al., 1992), implicating TR-specific effects are to be expected which can be predicted based on the outcome of TR-specific assays.

This, and the fact that exposure of organisms to compounds occurs in the presence of physiological background levels of T_3, illustrate the importance of testing the potential thyroid hormone disrupting effect of compounds in model systems that enable the characterization of the effects on both receptors independently.

Acknowledgements

The authors wish to thank prof. dr. Ivonne M.C.M. Rietjens (Wageningen University, Toxicology section, The Netherlands) for provision of helpful comments on the manuscript.

References

Adams, C., Lans, M.C., Klasson-Wehler, E., Engelen, van. J.G.M., Visser, T.J., Brouwer, A., 1990. Hepatic thyroid hormone 5'-deiodinase, another target-protein for monohydroxy metabolites of 3,3',4,4'-tetrachlorobiphenyl. **Organohalogen Compounds 1**, 51-54.

Bakker, O., Beeren van, H.C., Wiersinga, W.M., 1994. Desethylamiodarone is a noncompetitive inhibitor of the binding of thyroid hormone to the thyroid hormone beta 1-receptor protein. **Endocrinology 134**, 1665-1670.

Barrera-Hernandez, G., Park, K.S., Dace, A., Zhan, Q., Cheng, S.Y., 1999. Thyroid hormone-induced cell proliferation in GC cells is mediated by changes in G1 cyclin/cyclin-dependent kinase levels and activity. **Endocrinology 140**, 5267-5274.

Beeren van, H.C., Bakker, O., Wiersinga, W.M., 1995. Desethylamiodarone is a competitive inhibitor of the binding of thyroid hormone to the thyroid hormone alpha 1-receptor protein. **Mol Cell Endocrinol 112**, 15-19.

Bradley, D.J., Towle, H.C., Young, W.S., 1992. Spatial and Temporal Expression of Alpha-Thyroid and Beta-Thyroid Hormone Receptor Messenger-Rnas, Including the Beta-2-Subtype, in the Developing Mammalian Nervous-System. **J Neurosc 12**, 2288-2302.

Brasier, A.R., Tate, J.E., Habener, J.F., 1989. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cells. **Biotechniques 7**, 1116-1122.

Brouwer, A., Morse, D.C., Lans, M.C., Schuur, A.G., Murk, A.J., Klasson-Wehler, E., Bergman, A., Visser, T.J., 1998. Interactions of persistent environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. **Toxicol Indus Health 14**, 59-84.

Cheek, A.O., Kow, K., Chen, J., McLachlan, J.A., 1999. Potential mechanisms of thyroid disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. **Environ Health Persp 107**, 273-278.

Chiellini, G., Apriletti, J.W., Yoshihara, H.A., Baxter, J.D., Ribeiro, R.C., Scanlan, T.S., 1998. A high-affinity subtype-selective agonist ligand for the thyroid hormone receptor. **Chem Biol 5**, 299-306.

DeVito, M., Biegel, L., Brouwer, A., Brown, S., Brucker-Davis, F., Cheek, A. O., Christensen, R., Colborn, T., Cooke, P., Crissman, J. and others. 1999. Screening methods for thyroid hormone disruptors. **Environ Health Persp 107**, 407-415.

Furlow, J.D., Yang, H.Y., Hsu, M., Lim, W., Ermio, D. J., Chiellini, G., Scanlan, T.S., 2004. Induction of larval tissue resorption in Xenopus laevis tadpoles by the thyroid hormone receptor agonist GC-1. **J Biol Chem 279**, 26555-26562.

Gauger, K.J., Kato, Y., Haraguchi, K., Lehmler, H.J., Robertson, L.W., Bansal, R., Zoeller, R.T., 2004. Polychlorinated biphenyls (PCBs) exert thyroid hormone-like effects in the fetal rat brain but do not bind to thyroid hormone receptors. **Environ Health Persp 112**, 516-523.

Hamers, T., Kamstra, J.K., Sonneveld, E., Murk, A.J., Kester, M.H.A., Andersson, P.L., Legler, J., Brouwer, A., 2006. *In vitro* profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol Sci 92, 157-173.

Hoffer, A., Chang, C.Y., Puga, A., 1996. Dioxin induces transcription of fos and jun genes by Ah receptor-dependent and -independent pathways. Toxicol Appl Pharmacol 141, 238-247.

Ishihara, A., Sawatsubashi, S., Yamauchi, K., 2003. Endocrine disrupting chemicals: interference of thyroid hormone binding to transthyretins and to thyroid hormone receptors. **Mol Cell Endocrinol 199**, 105-117.

Kaltenbach, J. 1996. Endocrinology of Amphibian Metamorphosis. Gilbert L.I., Tata J.R., Atkinson B.G., editors. New York: Academic Press. 403 - 431 p.

Kitamura, S., Jinno, N., Ohta, S., Kuroki, H., Fujimoto, N., 2002. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. **Biochem Biophys Res Commun 293**, 554-559.

Laflamme, L., Hamann, G., Messier, N., Maltais, S., Langlois, M.F., 2002. RXR acts as a coregulator in the regulation of genes of the hypothalamo-pituitary axis by thyroid hormone receptors. **J Mol Endocrinol 29**, 61-72.

Lans, M.C., Klasson-Wehler, E., Willemsen, M., Meussen, E., Safe, S., Brouwer, A., 1993. Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-p-dioxins and -dibenzofurans with human transthyretin. **Chem Biol Interact 88**, 7-21.

Lans, M.C., Spiertz, C., Brouwer, A., Koeman, J.H., 1994. Different competition of thyroxine binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs and PCDFs. **Eur J Pharmacol 270**, 129-136.

Marsh, G., Bergman, A., Bladh, L.-G., Gillner, M., Jackobsson, E., 1998. Synthesis of p-Hydroxybromodiphenyl Ethers and binding to the thyroid receptor. **Organohalogen Compounds 37**, 305-309.

McKinney, J.D., Waller, C.L., 1994. Polychlorinated biphenyls as hormonally active structural analogues. **Environ Health Persp 102**, 290-297.

McKinney, J.D., Waller, C.L., 1998. Molecular determinants of hormone mimicry: halogenated aromatic hydrocarbon environmental agents. **J Toxicol Environ Health B Crit Rev 1**, 27-58.

Meerts, A.T.M., van Zanden, J.J., Luijks, E.A., van Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A., Brouwer, A., 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. **Toxicol Sci 56**, 95-104.

Moriyama, K., Tagami, T., Akamizu, T., Usui, T., Saijo, M., Kanamoto, N., Hataya, Y., Shimatsu, A., Kuzuya, H., Nakao, K., 2002. Thyroid hormone action is disrupted by bisphenol A as an antagonist. **J Clin Endocrinol Metab 87**, 5185-5190.

OECD Environment, Health and Safety publications, 2006. Draft detailed review paper on thyroid hormone disruption assays. Http://www.oecd.org/dataoecd/44/39/34822810.pdf.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: A laboratory Manual, e.d. 2. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Samuels, H.H., Stanley, F., Casanova, J., 1979. Depletion of L-3,5,3'-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. **Endocrinology 105**, 80-85.

Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H., Vennstrom, B., 1986. The C-Erb-a Protein Is a High-Affinity Receptor for Thyroid-Hormone. **Nature 324**, 635-640.

Schriks, M., Neff, E.S., Murk, A.J., Furlow, J.D., 2006a. Antagonistic and potentiating effects of BDE206 and Hexacyclododecane (HBCD) in a functional *in vivo* assay with Xenopus leavis tadpoles are comparable to earlier results observed in the T-screen. **Submitted to Toxicol letters.**

Schriks, M., Zvinavashe, E., Furlow, J.D., Murk, A.J., 2006b. Thyroid hormone-mediated disruption of tadpole tail regression by hexabromocyclododecane (HBCD) and 2,2',3,3',4,4',5,5',6-nona brominated diphenyl ether (BDE206). **Chemosphere, in press.**

Schriks, M., Vrabie, C.M., Gutleb, A.C., Faassen, E.J., Rietjens, I.M.C.M., Murk, A.J., 2006. T-screen to quantify functional potentiating, antagonistic and thyroid hormone-like activities of poly halogenated aromatic hydrocarbons (PHAHs). **Toxicol In Vitr**o 20, 490-498.

Schuur, A.G., Brouwer, A., Bergman, A., Coughtrie, M.W., Visser, T.J., 1998a. Inhibition of thyroid hormone sulfation by hydroxylated metabolites of polychlorinated biphenyls. **Chem Biol Interact 109**, 293-297.

Schuur, A.G., Legger, F.F., van Meeteren, M.E., Moonen, M.J., van Leeuwen-Bol, I., Bergman, A., Visser, T.J., Brouwer, A., 1998b. *In vitro* inhibition of thyroid hormone sulfation by hydroxylated metabolites of halogenated aromatic hydrocarbons. Chem Res Toxicol 11, 1075-1081.

Tata, J.R., 1993. Gene expression during metamorphosis: an ideal model for post-embryonic development. **Bioessays 15**, 239-248.

Weinberger, C., Thompson, C.C., Ong, E.S., Lebo, R., Gruol, D.J., Evans, R.M., 1986. The C-Erb-a Gene Encodes a Thyroid-Hormone Receptor. Nature 324, 641-646.

Wu, Y., Koenig, R.J., 2000. Gene regulation by thyroid hormone. **Trends Endocrinol Metab 11**, 207-211.

Yamada-Okabe, T., Sakai, H., Kashima, Y., Yamada-Okabe, H., 2005. Modulation at a cellular level of the thyroid hormone receptor-mediated gene expression by 1,2,5,6,9,10-

hexabromocyclododecane (HBCD), 4,4'-diiodobiphenyl (DIB), and nitrofen (NIP). **Toxicol Lett 155**, 127-133.

Yamauchi, K., Ishihara, A., Fukazawa, H., Terao, Y., 2003. Competitive interactions of chlorinated phenol compounds with 3,3',5-triiodothyronine binding to transthyretin: detection of possible thyroid-disrupting chemicals in environmental waste water. **Toxicol Appl Pharmacol 187**, 110-117.

Yamauchi, K., Prapunpoj, P., Richardson, S. J., 2000. Effect of diethylstilbestrol on thyroid hormone binding to amphibian transthyretins. **Gen Comp Endocrinol 119**, 329-339.

Yen, P.M., Wilcox, E.C., Hayashi, Y., Refetoff, S., Chin, W.W., 1995. Studies on the repression of basal transcription (silencing) by artificial and natural human thyroid hormone receptor-beta mutants. **Endocrinology 136**, 2845-2851.

Zoeller, R.T., 2005. Environmental chemicals as thyroid hormone analogues: New studies indicate that thyroid hormone receptors are targets of industrial chemicals? **Mol Cell Endocrinol 242**, 10-15.

4

Disruption of thyroid hormone-mediated *Xenopus laevis* tadpole tail tip regression by hexabromocyclododecane (HBCD) and 2,2',3,3',4'4',5,5',6-nona brominated diphenyl ether (BDE206)

Merijn Schriks[1], Elton Zvinavashe[1], J. David Furlow[2], Albertinka J. Murk[1]

[1]Division of Toxicology, Wageningen University, Wageningen, The Netherlands
[2]Dept. of Neurobiology, Physiology and Behavior, University of California, Davis, USA

Chemosphere in press

Abstract

Thyroid hormone regulates amphibian metamorphosis, including the transformation of a tadpole into a froglet and regression of the tail. *Xenopus laevis* tadpole tail tips in organ culture (*ex vivo*) undergo regression when exposed to 3,3′,5-triiodo-L-thyronine (T_3) and interference by chemicals with this process was utilized as a bioassay to detect thyroid hormone disruption. In the present study the bioassay was further validated by investigating its response to compound induced T_3-antagonism and –potentiation. Tadpole tail tips were exposed to two brominated flame retardants (BFRs) in presence or absence of T_3 at its EC_{50} (20 nM). T_3-induced tail tip regression was antagonized by 2,2′,3,3′,4,4′,5,5′,6-nona brominated diphenylether (BDE206) and potentiated by hexabromocyclododecane (HBCD) in a concentration dependent manner, which was consistent with results obtained with a *in vitro* T_3 dependent proliferation bioassay termed the T-screen. Neither compound induced any effect in the absence of T_3. The results indicate that studying possible hormone disrupting effects of agonistic, antagonistic or potentiating compounds should include combined exposure with the natural hormone at around its EC_{50} concentration. The results obtained with the tail tip exposures were in accordance with the T-screen predictions, and occurred at BFR-concentrations that were only 5-50 times those of T_3. The bioassay proved to be suitable not only for detecting T_3-agonism, but also for antagonism and potentiation.

Introduction

Brominated flame retardants (BFRs) are added to numerous consumer products such as electrical equipment, building material and textiles to make them more fire resistant (de Wit, 2002). The most used BFRs are brominated diphenylethers (BDEs), hexabromocyclododecane (HBCD), tetrabromobisphenol-A (TBBP-A) and brominated biphenyls (BBPs). Due to increasing evidence about their endocrine disrupting potency, the production of two commonly used BFRs, penta- and octaBDE, was ended in the European Union in 2004 (guideline 2003/11/EC). Two highly brominated flame retardants that are currently still produced are hexabromocyclododecane (HBCD) and 2,2',3,3',4,4',5,5',6,6'-decaBDE (BDE209). HBCD is a technical mixture of several isomers (10.3 % α-, 8.7 % β- and 81 % γ-isomer) and primarily used in polystyrene for thermal insulation in buildings. Currently, HBCD has been detected in practically all environmental matrices (Law et al., 2005) including human blood and breast milk (Thomsen et al., 2001; Covaci et al., 2003; Weiss et al., 2004). BDE209 is ubiquitous in the environment (de Boer, 2004; BSEF, 2004) and would be expected to be debrominated by abiotic environmental factors (Soderstrom et al., 2004), micro-organisms (Gerecke et al., 2005) or aquatic biota (Kierkegaard et al., 1999; La Guardia et al., 2004; Stapleton et al., 2004) to lower brominated BDEs including 2,2',3,3',4,4',5,5',6-nonaBDE (BDE206).

Several BDEs and their hydroxylated metabolites have been shown to be potent competitors with thyroxin (T_4) for binding to the plasma transport protein transthyretin (Meerts et al., 2000) and some of them directly bind to the Thyroid hormone Receptors (TRs) (Marsh et al., 1998; Cheek et al., 1999), all possibly resulting in a disturbed thyroid hormone (TH) homeostasis. Recently we studied the possible thyroid hormone disrupting effects of BFRs in an *in vitro* bioassay termed the T-screen (Schriks et al., 2006a). This assay employs a rat pituitary (GH_3) tumor cell line (Gutleb et al., 2005), the proliferation of which is dependent on TH and mediated through the TRs. In these studies HBCD and BDE206 respectively potentiated and antagonized T_3-dependent cell proliferation. Since *in*

vitro responses of compounds in an immortalized tumor cell line may be very different from those in natural primary cells or tissues, the aim of the present study was to investigate the thyroid hormone disrupting effects of HBCD and BDE206 in primary tissue and compare the responses to the *in vitro* T-screen. To reach this aim, freshly isolated *Xenopus laevis* tadpole tail tips in organ culture were exposed to HBCD or BDE206 in absence and presence of T_3. Tadpole tail tips cultured *ex vivo* respond directly and independently to T_3 by undergoing TR-mediated regression (Shaffer, 1963; Tata, 1966; Furlow et al., 2004). This experimental setup ascertains TH specificity of the tested compounds, since factors such as hormonal feed-back mechanisms, biotransformation and other hormones including corticoids that may also influence tail tip regression (Hayes, 1997), can be excluded. We report here the effects of HBCD and BDE206 on T_3-induced tadpole tail tip regression and compare the results to the *in vitro* T-screen. The results obtained also validate the bioassay for detection of not only T_3-(ant)agonism (Lim et al., 2002; Furlow et al., 2004) but also of T_3-potentiation by environmental contaminants.

Animals, Materials and Methods

Compounds

The two BFRs tested were obtained within the framework of the EU-funded project 'Flame retardants Integrated Risk assessment for Endocrine effects (FIRE)'. Hexabromocyclododecane (HBCD technical mixture, no data on impurities supplied by the provider) was obtained through BSEF (Brussels, Belgium) and 2,2',3,3',4,4',5,5',6-nonaBDE (BDE206 >99 %; Christiansson et al., 2006) was a kind gift from prof. Åke Bergman (Stockholm University, Sweden). HBCD, BDE206 and 3,3',5-triiodo-L-thyronine (T_3 ≥ 98 %; Sigma) (Figure 1) were dissolved in dimethylsulfoxide (DMSO, 99.9 %; Acros chemicals, Belgium) and stored at –80°C (T_3) or in the dark at room temperature.

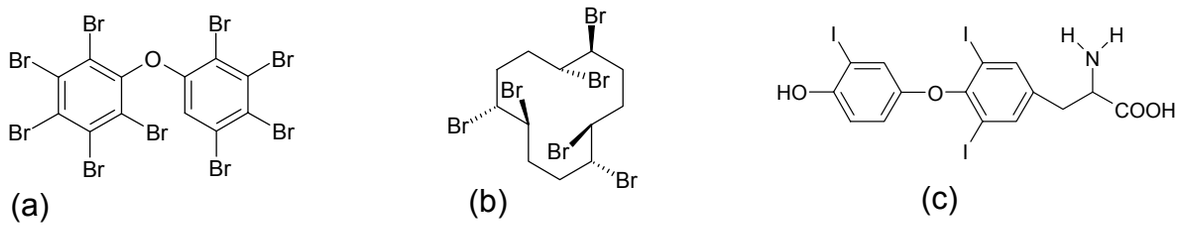


Figure 1. Chemical structures of (a) 2,2',3,3',4,4',5,5',6-nonaBDE (BDE206) (b) hexabromocyclododecane (HBCD) and (c) 3,3',5-triiodo-L-thyronine (T_3).

Exposure of tail tips to compounds

Premetamorphic *Xenopus laevis* tadpoles in developmental stage 52-53 (Nieuwkoop and Faber, 1975) (further abbreviated as NF) were purchased from Nasco, Inc (WI, USA). To arrest tadpoles in NF stage 53-54, methimazole (1-methyl-2-imidazolethiol, Sigma) was added to the rearing water at a concentration of 1 mM (Buckbinder and Brown, 1993) and water was changed every week. Methimazole (a goitrogen) interrupts TH synthesis and tadpoles can be kept in methamizole for months without adverse effects or altered behavior (Elinson et al., 1999). All animals were treated in accordance with an approved institutional animal use and care protocol.

Tadpole tail tips were cultured *ex vivo* as described (Furlow, et al., 2004) with modifications. Briefly, NF stage 53-54 tadpoles were pre-treated in Steinberg's solution (60 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 10 mM Hepes, pH 7.4) (Peng, 1991) containing 70 $mg.L^{-1}$ gentamycin and 200 $mg.L^{-1}$ streptomycin 24 h before removal of tail tips. Animals were anaesthetized in water containing 0.01 % MS-222 (buffered with 0.01 % $NaHCO_3$), after which 6 to 8 mm of the tail tip was cut (~15-20 % of whole tail length) and placed in 1 mL Steinberg's solution supplemented with 5 $\mu g.mL^{-1}$ insulin in 24 wells plastic culture dishes. Tail tips were left in culture media to stabilize for 24 h prior to exposure to compounds and all exposures were performed in six-fold. Compounds in combination with T_3 at its EC_{50} (20 nM; data derived from concentration response curve as presented by Furlow et al., 2004) were pre-mixed

in 1 mL Steinberg's medium and gently added to tail tips after removal of the old medium every other day.

The length of the tail tips was measured during six consecutive days using a millimetre grid. Based on results of pilot experiments, the tail tips were either exposed to DMSO (solvent control), T_3 at its EC_{50} (20 nM) or T_3 at its maximum effect concentration (100 nM) (Furlow et al., 2004), or to a combination of 20 nM T_3 together with BDE206 (1, 10, 100 or 1000 nM), or 20 nM T_3 together with HBCD (10, 100, 1000 or 10000 nM), or to 1000 nM BDE206 or 1000 or 10000 nM HBCD alone. The DMSO concentration in each well was kept < 0.5 % (v/v).

Statistical analysis

Combinatory exposures were all compared to the 20 nM T_3-control. Statistical analysis of the data was performed using SPSS version 10.1. Multiple comparison analysis was carried out using Bonferroni ($p \leq 0.05$) after testing for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test). No data transformation was necessary.

Results

During the 7 days that tail tips were kept in culture, no negative effects such as (fungal) infections were apparent. Exposure of tail tips to 100 nM T_3 resulted in 50 % regression after roughly 3 d. Tail tips exposed to 20 nM T_3 showed some variance in regression between experiments and 50 % regression occurred after 3.5 d and 5 d in the BDE206- and HBCD-experiment, respectively (Figure 2a/b). Tail tips exposed to the DMSO solvent control, showed some slight aspecific regression. However, this was not significant different from tail tips treated with compounds only (Figure 2a/b).

BDE206

On day 4, all BDE206 exposure concentrations in combination with 20 nM T_3 significantly reduced ($p < 0.05$) tail tip regression compared to the 20 nM T_3-control (Figure 2a). After 6 d of exposure this antagonistic effect could only still be observed in the 100 nM BDE206 (34 ± 4 %; $p \leq 0.05$) and 1000 nM BDE206 (46 ± 3 %; $p \leq 0.05$) exposure groups, relative to the 20 nM T_3-control. BDE206 alone (1000 nM) did not have any effect on tail tip regression.

HBCD

On day 6, exposure of tail tips to 1000 nM HBCD in combination with 20 nM T_3, significantly ($p \leq 0.05$) potentiated tail tip regression with 35 ± 5 % relative to the 20 nM T_3-control (Figure 2b). All lower HBCD exposures, including HBCD alone (1000 nM), did not have any effects on tail tip regression. Interestingly, 10 μM HBCD alone or in combination with 20 nM T_3 resulted in a very fast regression of tail tips in the first two days of exposure. This was faster than tail tip regression in the 100 nM T_3-control, but after two days of exposure tail tip regression roughly stayed the same during the rest of the experimental period.

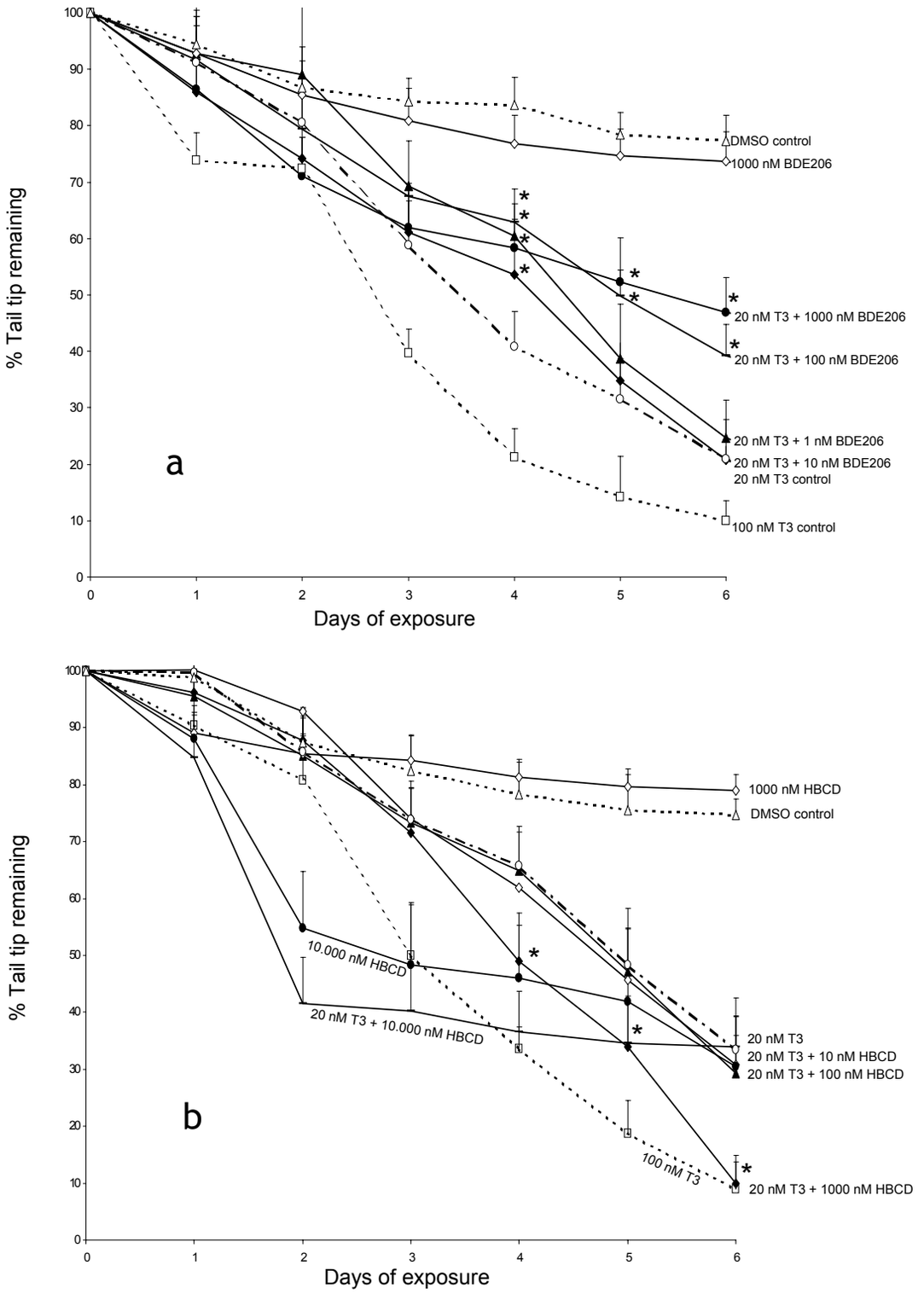


Figure 2. *Xenopus laevis* tadpole tail tips (NF 53-54) were exposed for 6 d to the DMSO control only (< 0.5 %, v/v), 20 nM T_3 (dotted line), 100 nM T_3 or an increasing concentration (a) BDE206 or (b) HBCD, co-administered with 20 nM T_3. All values are expressed as percentage of the initial tail tip length (set at 100 %), N=6. Error bars indicate S.D. *Significantly different from the 20 nM T_3-control ($p \leq 0.05$).

Discussion

The potentiating effects of HBCD and antagonistic effects of BDE206 on T_3-induced tail tip regression were in accordance with earlier results we obtained in the T-screen (Schriks et al., 2006a). Significant effects of BDE206 and HBCD on T_3-induced tail tip regression were observed at 100 nM and 1000 nM, respectively, which is only 5 - 50 times higher than the EC_{50} of T_3 (20 nM). In the T-screen, however, significant effects of both BFRs were observed at 100 nM, and this concentration was 400 times higher than the EC_{50} of T_3 (0.25 nM) in this *in vitro* system. This illustrates that higher concentrations BFRs are required for significant effects on T_3-induced tail tip regression, but that the concentration relative to that of T_3 (5 – 50 fold) is much lower than in the *in vitro* T-screen (400 fold). The effects on tail tip regression that occurred at a concentration of 10 μM HBCD, suggest that HBCD at this concentration may have cytotoxic activity. This hypothesis is supported by observations of Wang and Brown (1993) showing that T_3-induced tail resorption needs about a 2 day lag period before visible regression occurs. The variation in response of the 20 nM T_3-controls as observed between both experiments is a normally occurring phenomenon possibly due to a varying genetic background of the tadpoles, slight differences in tadpole developmental stages at the start of the experiment and slight differences in ambient temperature. Therefore it is always important to include the necessary DMSO- and T_3-controls in each experiment to relate the effects to, as was done in the present studies.

The mechanism of action of the BFRs tested in this study is not yet known. Recently, BDE206 has been shown to antagonize T_3-action in a TR isoform (α/β) selective transient reporter gene assay (Schriks et al., subm. 2006b), but did not compete with thyroxin (T_4) on binding to thansthyretin (TTR) (Hamers et al., 2006). HBCD on the other hand, potentiated T_3-action in the present study and in the T-screen, but did not have any effect in the T_4-TTR binding assay nor in the TR isoform selective reporter gene assay. The latter bioassay employs an African green monkey kidney fibroblast cell line (CV-1) that does not express endogenous

TRs (Yen et al., 1995). Therefore this system is more artificial than primary tail tissues and the T-screen which do possess endogenous TRs. However, HBCD potentiated human TRα mediated gene expression in a HeLaTR reporter gene assay (Yamada-Okabe et al., 2004). These results suggest that the tested BFRs interact at different levels of TR-mediated TH-signalling. Possible mechanisms of action may include interaction with various co-factors such as N-CoR or SRC-1 (Zoeller et al., 2005) or interference with the TR-RXR heterodimer complex. For example, 4'-hydroxy-2,3,3',4,5-pentachlorinated biphenyl (PCB106-OH) has been shown to disturb TRβ binding to a TR response element (TRE) because the TR-RXR heterodimer complex was partially dissociated from the TRE in the presence of PCB106-OH (Miyazaki et al., 2004).

The results presented in this paper demonstrate that two environmentally relevant BFRs namely HBCD and BDE206, respectively potentiate and antagonize a highly specific TH-regulated developmental program in *X. laevis* tadpoles, namely the resorption of primary tissues in organ culture. The effects observed occurred at concentrations that were only 5 to 50 times higher than T_3. Further investigation has shown that the effects induced by HBCD and BDE206 also occur in an *in vivo* immuno proliferation bioassay with one-week old *X. laevis* tadpoles (Schriks et al., subm. 2006c).

Finally, it can be concluded that the effects on tadpole tail tip regression occurred as predicted from the results in the *in vitro* T-screen, when tested in combination with the EC_{50} of the natural hormone. This further validates the bioassay and shows that it is able to detect not only T_3-(ant)agonism but also potentiation.

Acknowledgements

The helpful comments on the manuscript as provided by prof. dr. Ivonne M.C.M. Rietjens (Wageningen University, Toxicology section, The Netherlands) are gratefully acknowledged. This research was partially financed by a CEFIC-EMSG grant (project coordinator Christ de Rooij).

References

Boer de, J., 2004. Brominated flame retardants in the environment - the price for our convience. **Environ chem 1**, 81-85.

Bromine Science and Environmental Forum (BSEF), 2004. Fact Sheet DecaBDE. www.BSEF.com.

Buckbinder, L., Brown, D.D., 1993. Expression of the *Xenopus Laevis* Prolactin and Thyrotropin Genes during Metamorphosis. **P Natl Acad Sci USA 90**, 3820-3824.

Covaci, A., Voorspoels, S., de Boer, J., 2003. Determination of brominated flame retardants, with emphasis on polybrominated diphenyl ethers (PBDEs) in environmental and human samples - a review. **Environ Int 29**, 735-756.

Cheek, A.O., Kow, K., Chen, J., McLachlan, J.A., 1999. Potential mechanisms of thyroid hormone disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. **Environ Health Persp 107**, 273-278.

Christiansson, A., Teclechiel, D., Eriksson, E., Bergman, A., Marsh, G., 2006. Methods for the synthesis of nonabromodiphenyl ethers and a chloro-nonabromodiphenyl ether. **Chemosphere 63**, 562-569.

Elinson, R.P., Remo, B., Brown, D.D., 1999. Novel structural elements identified during tail resorption in *Xenopus laevis* metamorphosis: Lessons from tailed frogs. **Dev Biol 215**, 243-252.

Furlow, J.D., Yang, H.Y., Hsu, M., Lim, W., Ermio, D.J., Chiellini, G., Scanlan, T.S., 2004. Induction of larval tissue resorption in *Xenopus laevis* tadpoles by the thyroid hormone receptor agonist GC-1. **J Biol Chem 279**, 26555-26562.

Gerecke, A.C., Hartmann, P.C., Heeb, N.V., Kohler, H.P.E., Giger, W., Schmid, P., Zennegg, M., Kohler, M., 2005. Anaerobic degradation of decabromodiphenyl ether. **Environ Sci Technol 39**, 1078-1083.

Guardia la, M.J., Hale, R.C., Harvey, E., 2004. Environmental debromination of decabrominated diphenyl ether. Proc. The Third International Workshop on Brominated Flame Retardants (BFR2004), University of Toronto, Toronto, Ontario, Canada.

Gutleb, A.C., Meerts, I.A.T.M., Bergsma, J.H., Schriks, M., Murk, A..J., 2005. T-screen as a tool to identify thyroid hormone receptor active compounds. **Environ Toxicol Pharm 19**, 231-238.

Hamers, T., Kamstra, J.H., Sonneveld, E., Murk, A.J., Kester, M.H.A., Andersson, P.L., Legler, J., Brouwer, A., 2006. *In vitro* profiling of the endocrine disrupting potency of brominated flame retardants. **Toxicol Sci 92**, 157-173.

Hayes, T.B., 1997. Steroid as potential modulators of thyroid hormone activity in anuran metamorphosis. **Am Zool 37**, 185-194.

Kierkegaard, A., Balk, L., Tjarnlund, U., De Wit, C.A., Jansson, B., 1999. Dietary uptake and biological effects of decabromodiphenyl ether in rainbow trout (Oncorhynchus mykiss). **Environ Sci Technol 33**, 1612-1617.

Law, R.J., Kohler, M., Heeb, N.V., Gerecke, A.C., Schmid, P., Voorspoels, S., Covaci, A., Becher, G., Janak, K., Thomsen, C., 2005. Hexabromocyclododecane challenges scientists and regulators. **Environ Sci Technol 39**, 281A-287A.

Lim, W., Nguyen, N.-H., Yang, H.Y., Scanlan, T.S., Furlow, J.D., 2002. A thyroid hormone antagonist that inhibits thyroid hormone action *in vivo*. J Biol Chem 277, 35664-35670.

Marsh, G., Bergman, A., Bladh, L.-G., Gillner, M., Jackobsson, E., 1998. Synthesis of p-Hydroxybromodiphenyl Ethers and binding to the thyroid receptor. **Organohalogen Compounds 37**, 305-309.

Meerts, I.A., van Zanden, J.J., Luijks, E.A., van Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A., Brouwer, A., 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. **Toxicol Sci 56**, 95-104.

Miyazaki, W., Iwasaki, T., Takeshita, A., Kuroda, Y., Koibuchi, N., 2004. Polychlorinated biphenyls suppress thyroid hormone receptor-mediated transcription through a novel mechanism. J **Biol Chem 279**, 18195-18202.

Nieuwkoop, P. D. and Faber, J., 1975. Normal table of *Xenopus laevis*. Amsterdam, The Netherlands: North Holland Publishing.

Peng, H.B. 1991. Solutions and protocols. Kay, B.K. and Peng, H.B., editors. San Diego: Academic Press. 657–662.

Schriks, M., Vrabie, C.M., Gutleb, A.C., Faassen, E.J., Rietjens, I.M.C.M., Murk, A.J., 2006a. T-screen to quantify functional potentiating, antagonistic and thyroid hormone-like activities of poly halogenated aromatic hydrocarbons (PHAHs). **Toxicol in Vitro 20**, 490-498.

Schriks, M., Roessig, J.M., Murk, A.J., Furlow, J.D., 2006b. A transient reporter gene assay to identify thyroid hormone receptor isoform selectivity (TRα/β) of environmental contaminants. **Submitted to Environ Toxicol Phar.**

Schriks, M., Neff, E.S., Rietjens, I.M.C.M., Murk, A.J., Furlow, J.D., 2006c. *In vivo* effects of nona-brominated diphenyl ether (BDE206) and hexabromocyclododecane (HBCD) on cell proliferation in *Xenopus laevis* tadpoles: comparison to an *in vitro* cell based assay. **Submitted to Toxicol lett.**

Shaffer, B.M., 1963. The isolated *Xenopus laevis* tail: a preparation for studying the central nervous system and metamorphosis in culture. **J Embryol Exp Morphol 11**, 77-90.

Soderstrom, G., Sellstrom, U., de Wit, C.A., Tysklind, M., 2004. Photolytic debromination of decabromodiphenyl ether (BDE 209). **Environ Sci Technol 38**, 127-132.

Stapleton, H.M., Alaee, M., Letcher, R.J., Baker, J.E., 2004. Debromination of the flame retardant decabromodiphenyl ether by juvenile carp (Cyprinus carpio) following dietary exposure. **Environ Sci Technol 38**, 112-119.

Tata, J.R., 1966. Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. **Dev Biol 13**, 77-94.

Thomsen, C., Lundanes, E., Becher, G., 2001. Brominated flame retardants in plasma samples from three different occupational groups in Norway. **J Environ Monit 3**, 366-370.

Wang, Z., Brown, D.D., 1993. Thyroid Hormone-Induced Gene-Expression Program for Amphibian Tail Resorption. **J Biol Chem 268**, 16270-16278.

Weis, J.S., Meijer, L., Sauer, P., Linderholm, L., Athanassiadis, I., Bergman, A., 2004. PBDE and HBCDD levels in blood from Dutch mothers and infants - Analysis of a Dutch Groningen infant cohort. **Organohalogen Compounds 66.**

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

Yamada-Okabe, T., Aono, T., Sakai, H., Kashima, Y., Yamada-Okabe, H., 2004. 2,3,7,8-tetrachlorodibenzo-p-dioxin augments the modulation of gene expression mediated by the thyroid hormone receptor. **Toxicol Appl Pharmacol 194**, 201-210.

Yen, P.M., Wilcox, E.C., Hayashi, Y., Refetoff, S., Chin, W.W., 1995. Studies on the repression of basal transcription (silencing) by artificial and natural human thyroid hormone receptor-beta mutants. **Endocrinology 136**, 2845-2851.

Zoeller, R.T., 2005. Environmental chemicals as thyroid hormone analogues: New studies indicate that thyroid hormone receptors are targets of industrial chemicals? **Mol Cell Endocrinol 242**, 10-15.

5

In vivo effects of nona brominated diphenylether (BDE206) and hexabromocyclododecane (HBCD) on cell proliferation in *Xenopus laevis* tadpoles: comparison with an *in vitro* cell based assay

Merijn Schriks[1], Eric S. Neff[2], Ivonne M.C.M Rietjens[1], Albertinka J. Murk[1] J. David Furlow[2]

[1]Division of Toxicology, Wageningen University, Wageningen, The Netherlands
[2]Dept. of Neurobiology, Physiology and Behavior, University of California, Davis, USA

Abstract

Previously, we demonstrated that certain compounds affect Thyroid hormone Receptor (TR) mediated cell proliferation in an *in vitro* bioassay termed the T-screen. To examine whether the same compounds elicit a comparable response *in vivo*, we applied an *in vivo* proliferation assay using one-week old *Xenopus laevis* tadpoles. Triido-L-thyronine (T_3) induced cell proliferation in tadpole tissues is quantified with a fluorescent antibody against the phospho-histone H3 mitosis marker and image analysis. Cell proliferation in brain and the rostral head region responded to T_3 in a dose-dependent fashion. The dose response curve is less steep in the brain and therefore more suitable for further *in vivo* testing of compounds. In addition, T_3-induced cell proliferation in brain also correlated better (r^2=0.97) with effects of T_3 as observed in the T-screen. The effects of two model compounds on T_3-induced cell proliferation in brain of *Xenopus laevis* tadpoles were then characterized. Hexabromocyclododecane (HBCD) potentiates T_3-induced cell proliferation in the brain, whereas nona brominated diphenylether 206 (BDE206) had no effect. To investigate this discrepancy, the effect of BDE206 was also quantified in the rostral head region, and in this tissue BDE206 acted as an antagonist as expected. This may be best explained by a limited capacity for the highly lipophilic BDE206 to efficiently pass the blood brain barrier. We conclude that compounds such as HBCD and BDE206 may interfere with T_3-dependent development of vertebrates.

Introduction

During the last decade, the development of assays for testing specific mechanisms of toxicity has gained considerate attention. Important steps have been made in the development of *in vitro* toxicity tests including rapid and highly specific reporter gene assays (Murk et al., 1996; Legler et al., 1999; Blankvoort et al., 2001) or cell proliferation based assays (Soto et al., 1995; Gutleb et al., 2005). This has been especially evident in the field of endocrine disruption. Nevertheless, the exclusive use of *in vitro* assays often has limitations, such as an inability to metabolize chemicals whose activity is dependent on initial biotransformation. Although the predictive value of *in vitro* toxicity testing has greatly improved, the extrapolation to the *in vivo* situation remains difficult (Kremers, 2002). Since not all relevant mechanisms of action can be tested *in vitro*, additional *in vivo* testing is required to avoid false classification of a substance as lacking endocrine disrupting activity (EDSTAC, 1998).

Contrary to the sex hormone-axis, the effects of endocrine disrupting chemicals on the thyroid hormone (TH)-axis have not received so much attention, although it has been recognized that compounds can also interfere at different levels of this complex system (DeVito et al., 1999; Colborn, 2002). TH plays a crucial role in several processes in vertebrates such as regulating several metabolic pathways in mammals, birds and the development of neural tissues in all vertebrates, and postembryonic development of virtually every tissue and organ in amphibian tadpoles (Dickhoff et al., 1990). TH exerts its effects via the Thyroid hormone Receptors (TRs) and once occupied by TH they can influence gene transcription (Ichikawa and Hashizume, 1995; Zhang and Lazar, 2000). All vertebrates possess two highly conserved TR isoforms called TRα and TRβ (Yaoita et al., 1990) and it is currently believed that these different TR isoforms mediate both overlapping and distinct cellular functions (Gauthier et al., 1999). Although most attention on endocrine disruption is focused on the sex-steroid hormones, several *in vitro* assays have been developed to assess the impact of environmental contaminants on different levels of the TH-axis (Janosek et al., 2005). For example, *in vitro*

interactions of the polyhalogenated aromatic hydrocarbons (PHAHs) with the transport protein transthyretin (TTR) in blood have been reported (Brouwer et al., 1998; Brucker-Davis, 1998; Cheek et al., 1999; Meerts et al., 2000; Yamauchi et al., 2000; Yamauchi et al., 2003; Ishihara et al., 2003). Furthermore, the depletion of the colloidal T_4–ring has been recently described as a sensitive marker for thyroid hormone disruption (Mukhi et al., 2005; Hu et al., 2006).

More recently, we introduced the T-screen as a functional *in vitro* assay for TH disruption at the level of the TR (Gutleb et al., 2005; Schriks et al. 2006). This assay employs a rat pituitary cell line (GH_3), the cell proliferation of which is dependent on TH and mediated through the TRs. To address the *in vivo* effects of thyroid hormone disruption, amphibian postembryonic development has been proposed as an excellent model, since it is completely TH dependent and occurs independently of any maternal influence (Kanamori and Brown 1996; Tata, 1999; Shi, 2000). Most of the recently described amphibian based assays for TH disruption (Gutleb et al. 1999; Garber et al., 2001; Fort and Paul, 2002; Opitz et al., 2005) use morphological endpoints such as tail resorption or front limb emergence as a functional parameter. In many of these assays it is difficult to elucidate if compounds act via the thyroid hormone axis, or elicit their toxic action through alternative routes. In this study we present an assay to examine the effect of TH disrupting compounds on 3,3',5-triiodo-L-thyronine (T_3)-induced cell proliferation *in vivo*. Therefore one-week old *Xenopus laevis* tadpoles are exposed to compounds in the presence of T_3, and proliferating cells (mitotic phase) in different tissues are visualized using an antibody against the phospho-histone H3 mitosis marker (Schreiber et al., 2001). At this life stage tadpoles do not have any detectable endogenous TH (Leloup and Buscaglia, 1977), but are competent to respond to exogenous T_3 administration (Tata, 1968; Brown et al., 2005). This *in vivo* T_3-induced proliferation response offers a unique biomarker to investigate the effects of environmental contaminants on the cellular events induced by TH in different tissues. We compare the outcome of the *in vivo* proliferation assay with results obtained earlier with the *in vitro* T-screen (Schriks

et al., 2006). The model compounds applied are hexabromocyclododecane (HBCD) and nona brominated diphenylether 206 (BDE206). These compounds were selected because of their differential effect in the T-screen, HBCD showing a potentiating effect and BDE206 an antagonistic effect in the T-screen (Schriks et al. 2006). These compounds were also chosen for their environmental relevance. HBCD is primarily used as thermal insulator in buildings and is ubiquitous in the environment (Law et al., 2005). BDE206, an expected breakdown product of the flame retardant deca brominated diphenylether (BDE209) has been detected at concentrations up to 14.2 $nmol.kg^{-1}$ sediment (dry wt.) (Guardia et al., 2004; Soderstrom et al., 2004) and in serum of workers employed at an electronic recycling plant up to 0.73 $pmol.g^{-1}$ (lipid wt.) (Thuresson et al., 2006).

Animals, Materials and Methods

Chemicals

All chemicals were purchased from Sigma and of >98 % purity unless stated otherwise. For chemical treatments 3,3',5-triiodo-L-thyronine (T_3), hexabromocyclododecane (HBCD) and nona brominated diphenylether 206 (BDE206; bromine substitution pattern 2,2',3,3',4,4',5,5',6) were dissolved in dimethylsulfoxide (DMSO, 99.9 % pure; Acros chemicals, Geel, Belgium). T_3-stocks were stored at –80 °C, whereas other compounds were stored in the dark at room temperature. HBCD (technical mixture; no data on impurities provided by the supplier) was obtained through BSEF (Brussels, Belgium) and BDE206 (>99 %) was kindly supplied by prof. dr. Åke Bergman (Stockholm University, Sweden). The latter compounds were also tested in the framework of the EU-funded project Flame retardant Integrated Risk assessment for Endocrine effects (FIRE). The primary antibody (Ab) used in this study was a 1/300 diluted rabbit polyclonal against human phospho-histone H3 (Upstate biotechnology, Lake Placid, NY, USA) and the secondary Ab was 1/400 diluted anti-rabbit Alexa Fluor 488-conjugated (Molecular probes).

Animal studies

Xenopus laevis tadpoles were staged according to Nieuwkoop and Faber (NF) (Nieuwkoop and Faber, 1975) and fertilized embryos were obtained (animal facilities UC Davis, USA) as described previously (Schreiber et al., 2001), with some modifications. Briefly, 2 days before egg laying adult females received a 50 IU pregnant mare's serum gonadotropin injection into the dorsal lymph sac followed by a 500 IU human chorionic gonadotropin injection 12-14 hours prior to egg laying. Animals were kept in the dark until egg laying commenced. On the day of fertilization, one male was anesthetized in a buffered MS-222 solution (0.1 % w/v + 0.1 % $NaHCO_3$ w/v) after which the animal was decapitated and the testes removed on ice. Eggs were fertilized with macerated pieces of testes and de-jellied as described elsewhere (American Society for Testing and Materials, 1991). Embryos were raised in accordance with an approved institutional animal use and care protocol. For whole animal treatments, ten one-week old tadpoles (NF stage ~48) each measuring approx. 10 mm in length were placed in 55 mm plastic petridishes (Fisher) containing 20 mL 0.1 X Marc's modified ringers (MMR) solution (10 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.5 mM HEPES; pH 7.5) as described (Schreiber et al., 2001) and exposed to increasing concentrations (0 - 100 nM) of T_3 for the dose-response curves, or, in combination with 1 nM T_3, to 10, 100, 1000 nM and 10 μM HBCD or 10, 100 or 1000 nM BDE206. DMSO concentrations were kept ≤ 0.2 % and a DMSO solvent control was included in all experiments. The solutions were changed every other day and for practical reasons exposure lasted 6 and 8 days for BDE206 and HBCD respectively. Tadpoles were not fed during the exposure period and observed daily for mortality and/or malformations. After exposure the animals were fixed in a 4 % paraformaldehyde (PFA) solution in phosphate buffered saline (PBS) and further processed for immunocytochemistry as described in detail below.

Immunocytochemistry

Immunocytochemistry (ICC) was performed on tadpoles as described previously (Schreiber et al., 2001), with modifications. Briefly, tadpoles were collected in glass vials and fixed in 4 % PFA solution in PBS. Vials were kept in the dark on a nutator rocking action mixer (Clay Adams, further referred to as nutator) and incubated overnight at 4 °C. Tadpoles were than rinsed in PBS at least 3 times and cleared overnight in a 2:1 methanol:hydrogen peroxide (20 %) solution on a platform mixer (50-70 rpm) at room temperature in the dark. Animals were then permeabilized for three days by extensive washing on the nutator in 1 % Triton X-100 in PBS (PBT) at room temperature and left overnight at 4 °C. Immuno staining of whole tadpoles consisted of blocking in 10 % (v/v) goat serum for 4 hours, followed by an overnight incubation at 4 °C on the nutator in primary Ab diluted in PBT containing 10 % (v/v) goat serum. After an additional one hour incubation at room temperature, samples were rinsed extensively (at least 8 times) in PBT followed by the addition of secondary Ab diluted in PBT containing 10 % (v/v) goat serum and left overnight at 4 °C on the nutator. Following extensive washes in PBT (at least 8 times), samples were left on the nutator overnight at 4 °C. Proliferating cells were visualized with a Leica MZ FLIII fluorescence stereo dissection microscope equipped with a GFP2 filter set (a 480/40-nm excitation filter and a 510-nm barrier filter) and photographs were made with an Optronics LE750 digital camera. Immunocytochemical labeled proliferating cells (mitotic phase) in visual distinguishable tissues, including the rostral head region and brain, were quantified with the aid of Imagetool v3 for Windows (The University of Texas Health Science Center, San Antonio, TX, USA). The rostral head region was defined as the area in front of the brain that includes the majority of proliferating cells in the head, including the lower jaw and olfactory epithelia (Figure 1).

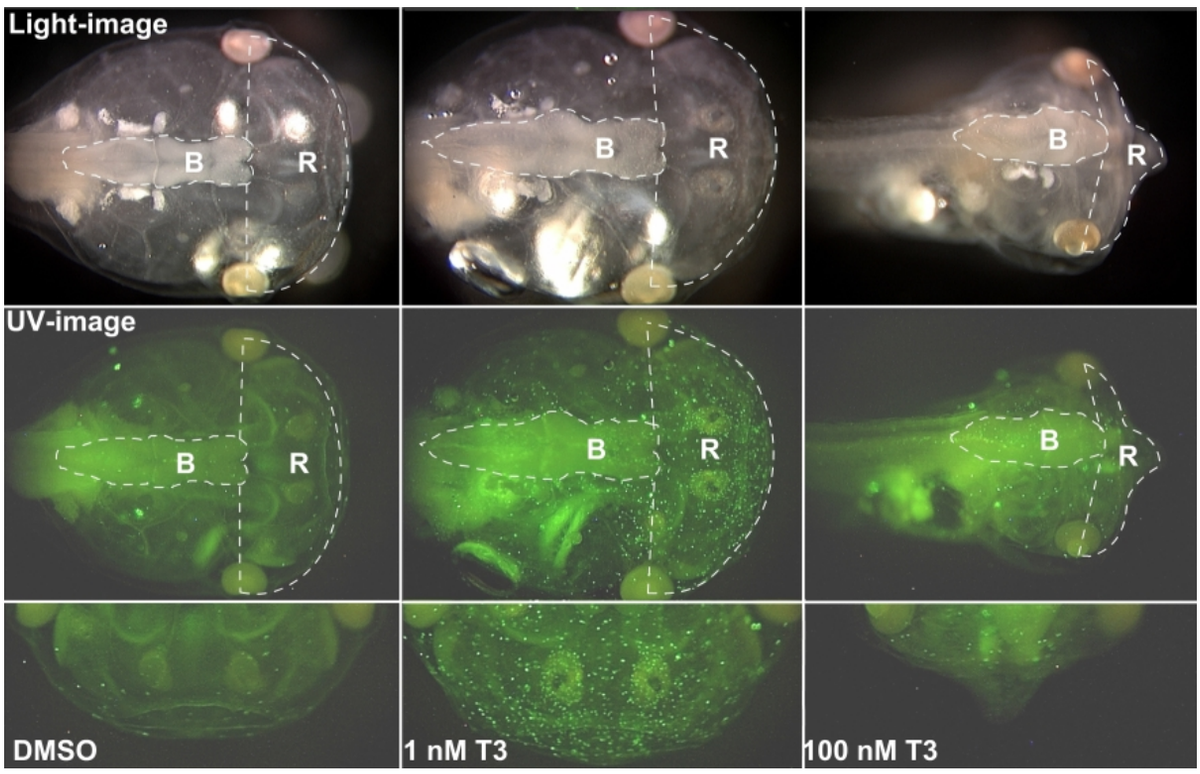


Figure 1. One-week old *Xenopus laevis* tadpoles were treated with 3,3',5-triiodo-L-thyronine (T_3) for 8 days. Proliferating cells were labeled by whole body immunofluorescence with an antibody against the phospho-histone H3 mitosis marker and pictures were taken as light image (top) or under UV-light (middle). Proliferating cells were counted in either B(rain) or R(ostral head) region. Pictures on the bottom represent magnified cut out of the rostral head region illustrating proliferating cells.

Statistical analysis

Statistical analyis of the data was carried out using the Student's *t*-test. The acceptance level was set at $p \leq 0.05$.

Results

In general no mortality occurred in any of the experimental groups and none of the animals showed malformations.

T_3 increases the number of proliferating cells in tadpole brain and the rostral head region in a dose-dependent fashion.

Figure 2 shows the effects of exposure to increasing concentrations of T_3 on the number of proliferating cells in brain and the rostral head region of one-week old *Xenopus laevis* tadpoles. After exposure to T_3 a dose related increase in the number of proliferating cells was observed both in the brain and the rostral head region, reading a maximum at 5 nM T_3 (227 ± 42) and 1 nM (368 ± 73) T_3, respectively. The EC_{50} value for the T_3-mediated effect on the number of proliferating cells in brain was 0.77 ± 0.31 nM T_3 and for the rostral head region 0.57 ± 0.19 nM T_3. As shown in Figure 2, the slope of the graph for the rostral head region was very steep, with a 5-fold difference between the EC_{10} and EC_{100}.

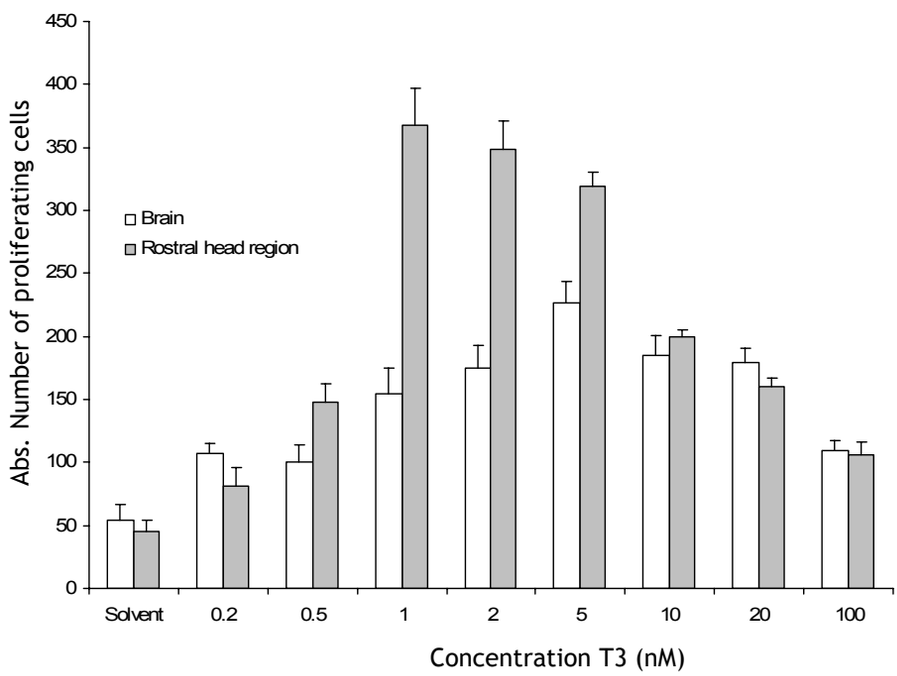


Figure 2. Induction of cell proliferation in brain and rostral head region of one-week old *Xenopus laevis* tadpoles. Animals were treated with increasing concentrations of T_3 for 8 days. Proliferating cells were visualized by whole-body immunofluorescence with an antibody against the phospho-histone H3 mitosis marker and counted in at least 6 different animals. Bars represent absolute numbers of proliferating cells in different tissues ± SEM.

For brain this difference was only 2.5-fold; therefore the dose-response curve for the brain is more suitable for quantifying interacting effects of T_3 and potentiating or (ant)agonistic compounds. Cell proliferation decreases at higher concentrations of T_3, possibly due to precocious differentiation of cells as observed in ecdysone induced proliferation of the Drosophila eye (Champlin and Truman, 1998) or increased apoptosis. After 8 days exposure to 100 nM T_3, animals have resorbed their gills completely (data not shown) and develop a protruding lower jaw (expansion of Meckel's cartilage) as described previously (Schreiber et al., 2001).

Effect of HBCD and BDE206 on T_3-induced cell proliferation in the brain.

Using brain as the target tissue, the effect of HBCD and BDE206 on the T_3-induced cell proliferation was characterized. Figure 3 reveals that exposure of tadpoles to 100 or 1000 nM HBCD in combination with 1 nM T_3, significantly ($p \leq 0.05$) potentiated the number of proliferating cells in the brain with 33.2 % and 24.5 % increase relative to the 1 nM T_3 control group. Compared to the solvent control, HBCD itself (1000 nM) has no effect on cell proliferation.

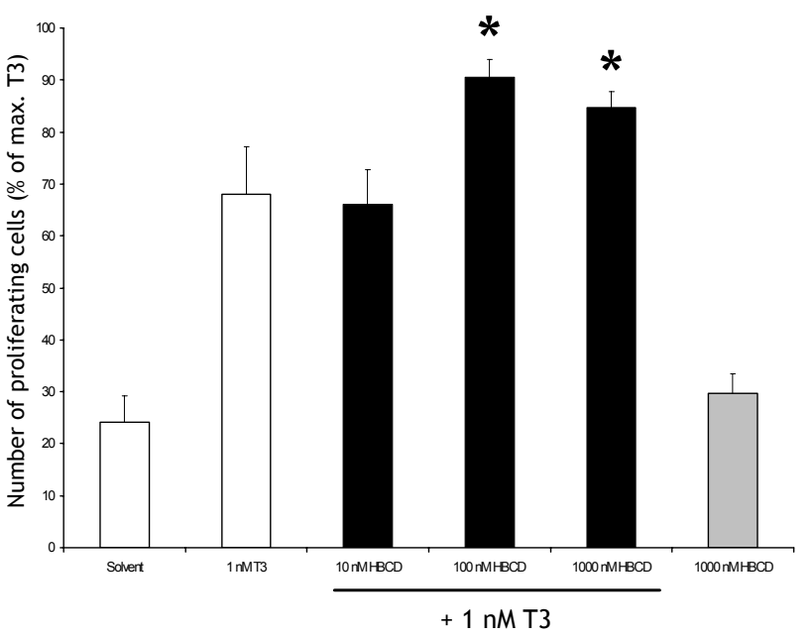


Figure 3. Effect of hexabromocyclododecane (HBCD) in combination with 1 nM T_3 on the number of proliferating cells in the brain of one-week old *Xenopus laevis* tadpoles. Proliferating cells were visualized after 8 days by whole-body immunofluorescence with an antibody against the phospho-histone H3 mitosis marker and counted in at least 6 different animals. Bars represent % of counted proliferating cells ± SEM relative to max. T_3 induction. *Significant different from the 1 nM T_3 control group ($p \leq 0.05$).

Figure 4 shows the effect of BDE206 on T_3-induced cell proliferation in the brain of one-week old *Xenopus laevis* tadpoles. BDE206 exposure of tadpoles in combination with 1 nM T_3, did not result in a significant effect on the T_3-induced cell proliferation.

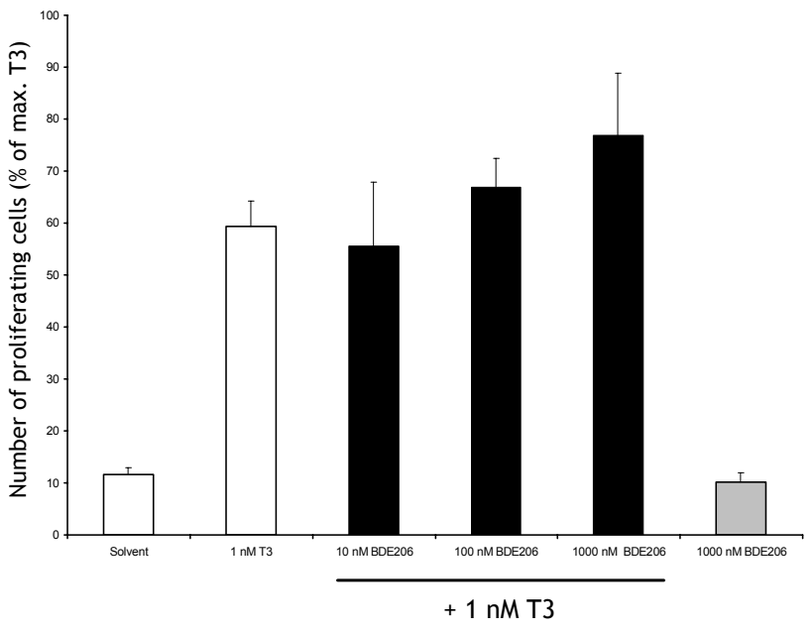


Figure 4. Effect of nona brominated diphenylether (BDE206) in combination with 1 nM T_3 on the number of proliferating cells in the brain of one-week old *Xenopus laevis* tadpoles. Proliferating cells were visualized after 6 days by whole-body immunofluorescence with an antibody against the phospho-histone H3 mitosis marker and counted in at least 6 different animals. Bars represent % of counted proliferating cells ± SEM relative to max. T_3 induction.

Effect of BDE206 on T_3-induced cell proliferation in the rostral head region.

BDE206 did not reveal an effect on T_3-induced cell proliferation in the brain, possibly due to the limited capacity of this lipophilic compound to pass the blood-brain barrier to a significant extent within 6 days. Therefore, the effect of BDE206 on T_3-induced cell proliferation in the rostral head region was quantified as well. Figure 5 shows the result obtained and reveals that BDE206 was able to reduce the number of proliferating cells induced by 1 nM T_3 in a dose-dependent fashion. Only the reduction of 20.3 % by 1000 nM BDE206 was statistically significant ($p < 0.05$) from the 1 nM T_3 control.

BDE206 exposure alone (1000 nM) did not significantly alter cell proliferation in the rostral head region of tadpoles.

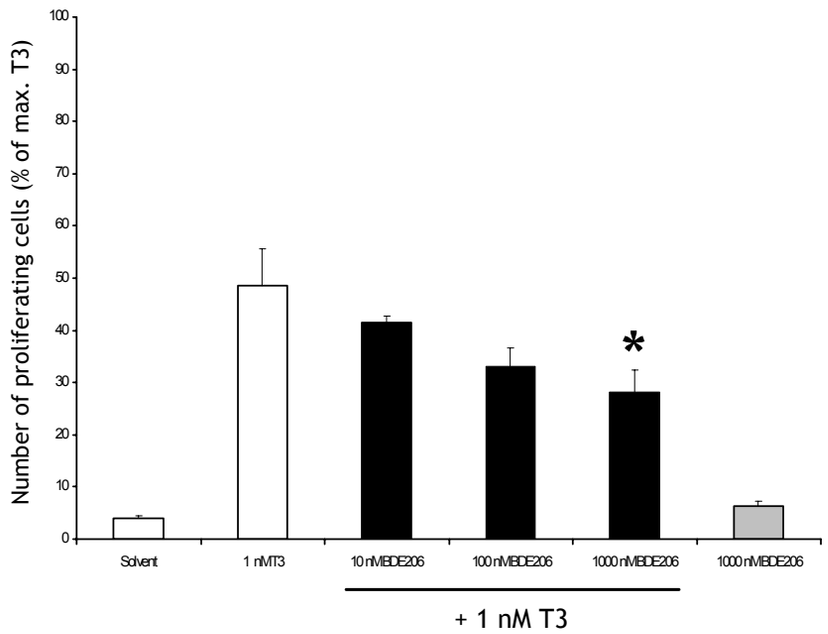


Figure 5. Effect of nona brominated diphenylether (BDE206) in combination with 1 nM T_3 on the number of proliferating cells in the rostral head region of one-week old Xenopus laevis tadpoles. Proliferating cells were visualized after 6 days by whole-body immunofluorescence with an antibody against the phospho-histone H3 mitosis marker and counted in at least 6 different animals. Bars represent % of counted proliferating cells ± SEM relative to max. T_3 induction. *Significant different from the 1 nM T_3 control group ($p < 0.05$).

Comparison of the in vivo bioassay results with those obtained previously for the in vitro cell based T-screen assay.

To validate the *in vivo* bioassay, the immunocytochemistry results were compared to those previously obtained *in vitro* using the T-screen. This comparison reveals a high correlation ($R^2 = 0.97$) between the effects of T_3 on cell proliferation in the tadpole brain and cell proliferation in the T-screen (Figure 6). The correlation between T_3-induced cell proliferation in the rostral head region of tadpoles and the T-screen is much lower ($R^2 = 0.47$; Figure not shown). These results illustrate that the *in vitro* T-screen has a high predictive value for the brain response in the *in vivo* proliferation assay.

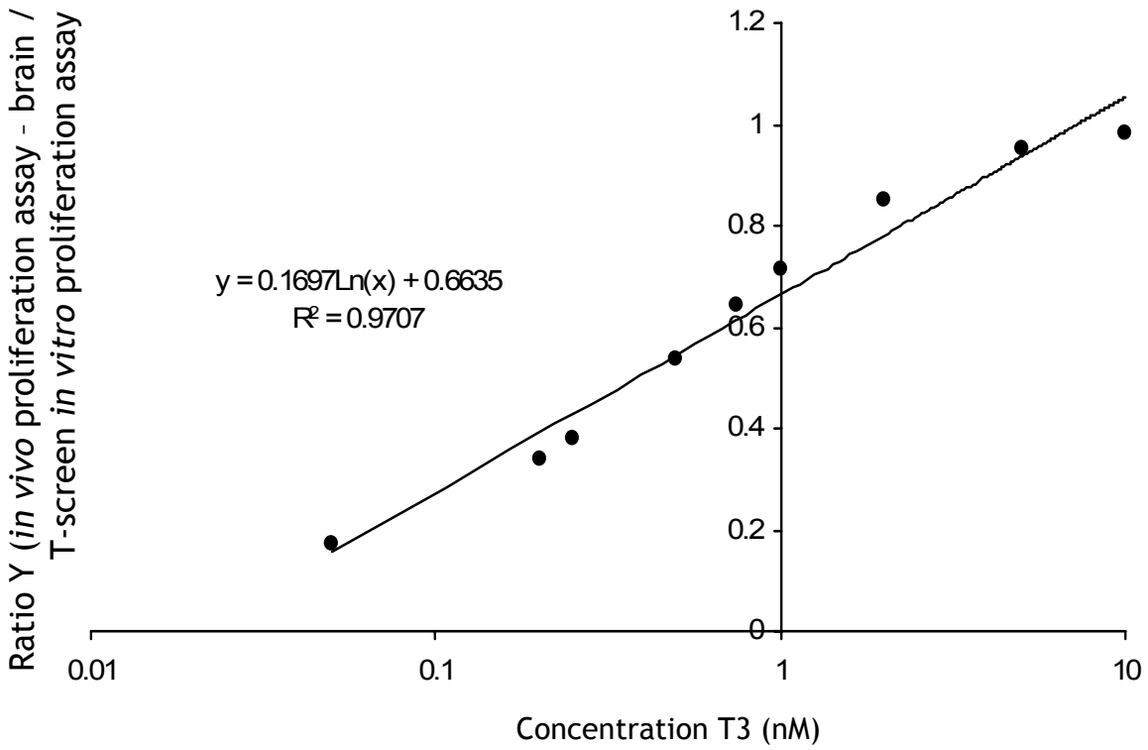


Figure 6. Correlation between the *in vivo* proliferation assay (brain) and the *in vitro* T-screen (Schriks et al., 2006). Data of T_3 dose-response curves (range 0-10 nM T_3) was fitted according to the general sigmoidal curve with hill slope model (y = a-d) / $(1+x/c)^b$ + d) (Life Science Workbench (LSW) Data Analysis Toolbox Version 1.11; MDL Information systems, San Leandro, CA, USA), resulting in the following parameters for each bioassay; T-screen, a=0, b=0.93, c=0.21, d=100; *in vivo* proliferation assay, a=0, b=1.20, c=0.77, d=100. After calculating the ratio y (*in vivo* proliferation assay) : y (T-screen) a regression equation of y = 0,017 Log10(x) + 0,0664 with R^2 = 0.97 was obtained.

Figure 7 compares the results obtained in the *in vivo* bioassay and the T-screen for the disrupting effects of the two model compounds HBCD and BDE206. For comparison the effect recently observed for these two model compounds in the *ex vivo* tail resorption test (Schriks et al., 2006) are also included in this figure. We can conclude that the *in vivo* bioassay of the present study is able to detect both the potentiating effect of HBCD as well as the antagonistic effect of BDE206. However, the absence of any effect of BDE206 in brain tissue points at a limited capacity of BDE206 to pass the blood-brain barrier and requires further optimization of target tissue specific exposure time in the *in vivo* bioassay.

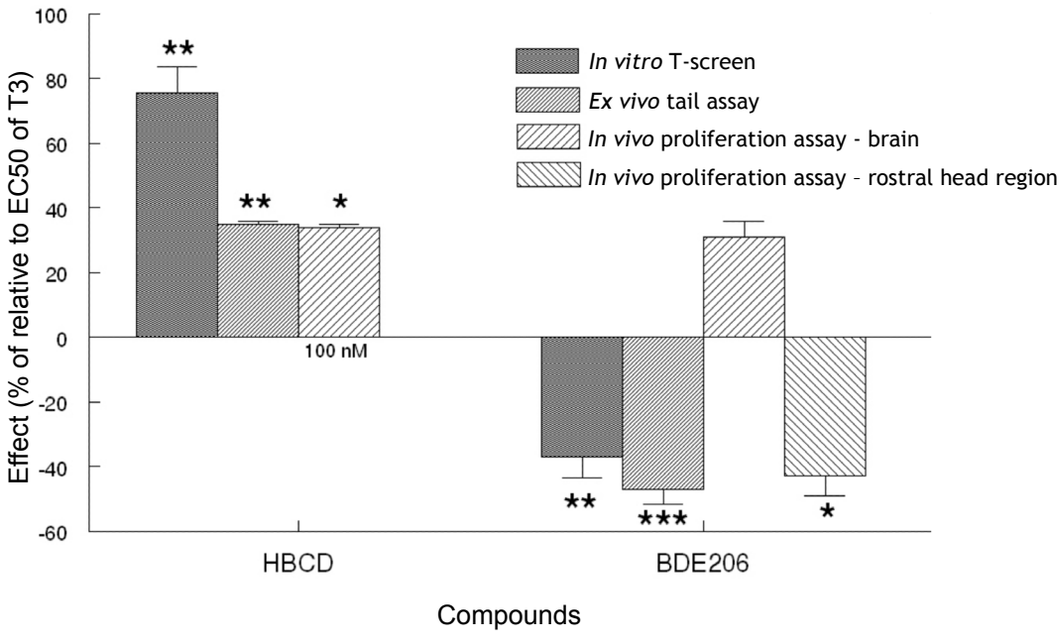


Figure 7. Effects of 1000 nM HBCD (8 days) and BDE206 (6 days) in combination with the EC_{50} of T_3 in various bioassays. HBCD in the *in vivo* proliferation assay (brain) has been depicted at 100 nM, since effects of this compound were maximal at that concentration. *Significant different from the EC_{50} of T_3 ($p < 0.05$); ** $p < 0.01$; *** $p < 0.001$.

Discussion

The aim of the present study was to validate the use of an *in vivo* bioassay for testing thyroid hormone endocrine disrupting effects, based on T_3-induced cell proliferation in *Xenopus laevis* tadpoles. To this end, the effects on T_3-induced cell proliferation in two target tissues and the effects of two model compounds were characterized. The results were compared to those previously obtained with an *in vitro* proliferation assay termed the T-screen (Schriks et al., 2006). For the *in vivo* bioassay one-week old *Xenopus laevis* tadpoles were exposed to 3,3',5-triiodo-L-thyronine (T_3) in combination with hexabromocyclododecane (HBCD) or nona brominated diphenylether (BDE206) and cell proliferation in brain and the rostral head region was visualized by immunocytochemistry and quantified by image analysis. A T_3-dose-dependent increase in cell proliferation in tadpole brain and the rostral head region was observed. The concentration dependent response in brain is not as steep and therefore more suitable for further *in vivo* testing of

compounds that may modulate T_3 dependent cellular events. The T_3-induced brain proliferation *in vivo* also correlated better (r^2=0.97) with the T_3 mediated effect on cell proliferation in the *in vitro* T-screen. After establishing reference values for T_3 response in brain and the rostral head region, the effects of HBCD and BDE206 were examined. HBCD at 100 and 1000 nM potentiates the T_3-induced cell proliferation in brain, an effect similar to what was previously observed in the *in vitro* T-screen. The effective concentration HBCD relative to T_3, was only a 100-fold higher than T_3 (100 nM to 1 nM) compared to 400-fold in the T-screen (100 nM to 0.25 nM) (Schriks et al., 2006).

BDE206 appeared to be inactive in the brain region whereas it was a potent T_3 antagonist in the T-screen. To investigate this apparent discrepancy in more detail, the effect of BDE206 on T_3-induced cell proliferation was also quantified in the rostral head region. This analysis revealed that in this tissue BDE206 indeed acts as an antagonist in that area of the tadpole. The antagonistic effects in the rostral head region were of the same order of magnitude as earlier observed in the T-screen (Schriks et al., 2006). The rostral head region may be exposed more easily to the lipophilic compounds resulting in higher tissue levels. Therefore, BDE206 may have a limited capacity to pass the blood-brain barrier and indicates the need for further optimization of target tissue specific exposure time in the *in vivo* assay. The latter because sufficient long exposure of accessible tissues such as the rostral head region to agonistic, potentiating or synergistic compounds including HBCD, may lead to a decrease in cell proliferation due to differentiation/apoptosis as seen at high T_3-concentrations (Figure 2). Therefore assay of the rostral head response may provide a preferable test tissue for highly lipophilic compounds that have difficulties in passing the blood-brain barrier. Further optimization of the possibly compound-specific tissue and exposure time could be of interest but was beyond the scope of the present study. Another reason to further study and optimize tissue specific responses is that molecular mechanisms through which selective effects are obtained may be revealed. Finally, to avoid misinterpretation

of the results due to temporary and spatial responses of the various tissues to T_3, it is important to repeat the experiments with the same exposure period.

Differential responses likely involve the expression of the specific thyroid hormone receptor (TR) isoforms TRα and TRβ in different tissues. According to Kawahara and co-workers (1991) the highest concentration of TRα is seen in tadpole brain, whereas TRβ is highly expressed in the head including the rostral head region. Although it has been reported that TRβ mRNA is very low in one-week old tadpoles (Yaoita and Brown, 1990), TRβ activation may become important for cell proliferation after several days of exogenous T_3 administration. Furthermore, cell-specific expression of nuclear receptor associated coactivator and corepressor proteins may also alter the responses to exogenous ligands and has been well-documented in other systems (Smith and O'Malley, 2004).

Interestingly, neither HBCD or BDE206 were able to affect cell proliferation *in vivo* in the absence of T_3. However, exposure to HBCD and BDE206 in the presence of T_3 more accurately reflects the real life situation. For tadpoles during natural metamorphosis and for humans, peak T_3 concentrations of respectively 18 nM and 26.4 nM have been reported (Henneman, 1986; Krain and Denver, 2004). Thus, in the presence of physiological concentrations T_3, these compounds may elicit their toxic action. HBCD has been detected at peak concentrations of 54.6 pM in serum of Dutch mothers (Weis et al., 2004) and (structurally unidentified) nona BDEs have been found in serum of Swedish computer technicians in levels up to 7 pM (Jacobsson et al., 2002). Since amphibian postembryonic development is reminiscent of human fetal and perinatal development, maternal transfer of toxic compounds leading to exposure and accumulation by the human fetus (Hites et al., 2003) may compromise normal development.

In conclusion, the *in vivo* proliferation assay in one-week old *Xenopus laevis* tadpoles provides a tissue specific *in vivo* bioassay with a high correlation to the responses in the *in vitro* T-screen. The exact mechanism of tissue specific effects remains to be elucidated, but must be taken into consideration in determining whether a chemical may act as an endocrine disrupting compound.

Acknowledgements

The authors wish to thank Jock Hamilton (University of California, Davis, USA) for technical assistance and dr. Timo Hamers (Institute for Environmental Studies, The Netherlands) for providing the compound stock solutions.

References

American Society for Testing and Materials, A., 1991. Standard guide for conducting the frog embryo teratogenesis assay-Xenopus. Annual book of ASTM standards 1-11.

Blankvoort, B. M. G., de Groene, E. M., van Meeteren-Kreikamp, A. P., Witkamp, R. F., Rodenburg, R. J. T., Aarts, J. M. M. J. G., 2001. Development of an androgen reporter gene assay (AR-LUX) utilizing a human cell line with an endogenously regulated androgen receptor. **Anal Biochem 298**, 93-102.

Brown, D. D., Cai, L. Q., Das, B., Marsh-Armstrong, N., Schreiber, A. M., Juste, R., 2005. Thyroid hormone controls multiple independent programs required for limb development in *Xenopus laevis* metamorphosis. **P Natl Acad Sci USA 102**, 12455-12458.

Brucker-Davis, F., 1998. Effects of environmental synthetic chemicals on thyroid function. **Thyroid 8**, 827-856.

Champlin, D. T., Truman, J. W., 1998. Edcysteriods govern two phases of eye development during metamorphosis of the moth, *Maduca sexta*. **Development 125**, 2009-2018.

Cheek, A. O., Kow, K., Chen, J., McLachlan, J. A., 1999. Potential mechanisms of thyroid disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. **Environ Health Persp 107**, 273-278.

Colborn, T., 2002. Clues from wildlife to create an assay for thyroid system disruption. **Environ Health Persp 110 Suppl 3**, 363-367.

DeVito, M., Biegel, L., Brouwer, A., Brown, S., Brucker-Davis, F., Cheek, A. O., Christensen, R., Colborn, T., Cooke, P., Crissman, J. and others, 1999. Screening methods for thyroid hormone disruptors. **Environ Health Persp 107**, 407-415.

Dickhoff, W. W., Brown, C. L., Sullivan, C. V., Bern, H. A., 1990. Fish and Amphibian Models for Developmental Endocrinology. **J Exp Zool suppl 4**, 90-97.

EDSTAC. 1998. Endocrine disruptor screening and testing advisory committee final report. http://www.epa.gov/scipoly/oscpendo/edspoverview/finalrpt.htm.

Fort, D. J., Paul, R. R., 2002. Enhancing the predictive validity of Frog Embryo Teratogenesis Assay - Xenopus (FETAX). **J Appl Toxicol 22**, 185-191.

Garber, E., Larsen, G. L., Hakk, H., A., B., 2001. Frog embryo teratogenic assay: Xenopus (FETAX) analysis of the biological activity of Tetra Bromo Bisphenol A (TBBPA). Abstracts of the 2nd International Workshop on Brominated Flame Retardants, 259-262.

Gauthier, K., Chassande, O., Plateroti, M., Roux, J. P., Legrand, C., Pain, B., Rousset, B., Weiss, R., Trouillas, J., Samarut, J., 1999. Different functions for the thyroid hormone receptors TRalpha and TRbeta in the control of thyroid hormone production and post-natal development. **Embo J 18**, 623-631.

Guardia, L. M. J., Hale, R. C., Harvey, E., 2004. Environmental debromination of decabrominated diphenyl ether. Proc. The Third International Workshop on Brominated Flame Retardants (BFR2004), University of Toronto, Toronto, Ontario, Canada.

Gutleb, A. C., Meerts, I. A. T. M., Bergsma, J. H., Schriks, M., Murk, A. J., 2005. T-Screen as a tool to identify thyroid hormone receptor active compounds. **Environ Toxicol Pharm 19**, 231-238.

Henneman, G., 1986. Thyroid hormone metabolism. Marcel Dekker, Inc., New York. ISBN 0-8247-7475-2.

Hites, R.A., Dodder, N.G., Mazdai, A., Abernathy, M.P., Bigsby, R.M., 2003. Polybrominated diphenylethers in maternal and fetal blood samples. **Organohalogen compounds 61**, 5-8.

Hu, F., Sharma, B., Mukhi, S., Patino, R., Carr, J.A., 2006. The colloidal thyroxine (T_4) ring as a novel biomarker of perchlorate exposure in the African clawed frog *Xenopus laevis*. **Toxicol Sci, in press.**

Ichikawa, K., Hashizume, K., 1995. Thyroid hormone action in the cell. **Endocrine J 42**, 131-140.

Ishihara, A., Sawatsubashi, S., Yamauchi, K., 2003. Endocrine disrupting chemicals: interference of thyroid hormone binding to transthyretins and to thyroid hormone receptors. **Mol Cell Endocrinol 199**, 105-117.

Jakobsson, K., Thuresson, K., Rylander, L., Sjödin, A., Hagmar, L., Bergman, A., 2002. Exposure to polybrominated diphenyl ethers and tetrabromobisphenol A among computer technicians. **Chemosphere 46**, 709-716.

Janosek, J., Hilscherova, K., Blaha, L., Holoubek, I., 2005. Environmental xenobiotics and nuclear receptors - Interactions, effects and *in vitro* assessment. **Toxicol in Vitro 20,** 18-37.

Kanamori, A., Brown, D. D., 1996. The analysis of complex developmental programmes: amphibian metamorphosis. **Genes Cells 1**, 429-435.

Kawahara, A., Baker, B. S., Tata, J. R., 1991. Developmental and Regional Expression of Thyroid-Hormone Receptor Genes during Xenopus Metamorphosis. **Development 112**, 933-943.

Krain, L. P., Denver, R. J., 2004. Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*. **J Endocrinol 181**, 91-104.

Kremers, P., 2002. Can drug-drug interactions be predicted from *in vitro* studies? **Scientific World Journal 2**, 751-766.

Law, R. J., Kohler, M., Heeb, N. V., Gerecke, A. C., Schmid, P., Voorspoels, S., Covaci, A., Becher, G., Janak, K., Thomsen, C., 2005. Hexabromocyclododecane challenges scientists and regulators. **Environ Sci Technol 39**, 281A-287A.

Legler, J., van den Brink, C. E., Brouwer, A., Murk, A. J., van der Saag, P. T., Vethaak, A. D., van der Burg, P., 1999. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. **Toxicol Sci 48**, 55-66.

Leloup, J., Buscaglia, M., 1977. Triiodothyronine, Hormone of Amphibian Metamorphosis. **Comptes Rendus Hebdomadaires Des Seances De L'Academie Des Sciences Serie D 284**, 2261-2263.

Li, A. P., Bode, C., Sakai, Y., 2004. A novel *in vitro* system, the integrated discrete multiple organ cell culture (IdMOC) system, for the evaluation of human drug toxicity: comparative cytotoxicity of tamoxifen towards normal human cells from five major organs and MCF-7 adenocarcinoma breast cancer cells. **Chem Biol Interact 150**, 129-136.

Meerts, I. A., van Zanden, J. J., Luijks, E. A., van Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A., Brouwer, A., 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. **Toxicol Sci 56**, 95-104.

Mukhi, S., Carr, J.A., Anderson, T.A., Patino, R., 2006. Novel biomarkers of perchlorate exposure in zebrafish. **Environ Toxicol Chem 24**, 1107-1115.

Murk, A. J., Legler, J., Denison, M. S., Giesy, J. P., van de Guchte, C., Brouwer, A., 1996. Chemical-activated luciferase gene expression (CALUX): a novel *in vitro* bioassay for Ah receptor active compounds in sediments and pore water. **Fund Appl Toxicol 33**, 149-160.

Nieuwkoop, P. D. and Faber, J., 1975. Normal table of *Xenopus laevis*. Amsterdam, The Netherlands: North Holland Publishing.

Opitz, R., Braunbeck, T., Bogi, C., Pickford, D. B., Nentwig, G., Oehlmann, J., Tooi, O., Lutz, I., Kloas, W., 2005. Description and initial evaluation of a Xenopus metamorphosis assay for detection of thyroid system-disrupting activities of environmental compounds. **Environ Toxicol Chem 24**, 653-664.

Schreiber, A. M., Das, B., Huang, H., Marsh-Armstrong, N., Brown, D. D., 2001. Diverse developmental programs of *Xenopus laevis* metamorphosis are inhibited by a dominant negative thyroid hormone receptor. **P Natl Acad Sci USA 98**, 10739-10744.

Schriks, M., Vrabie, C. M., Gutleb, A. C., Faassen, E. J., Rietjens, I. M. C. M., Murk, A. J., 2006. T-screen to quantify functional potentiating, antagonistic and thyroid hormone-like activities of poly halogenated aromatic hydrocarbons (PHAHs). **Toxicol in Vitro 20**, 490-498.

Schriks, M., Zvinavashe, E., Furlow, J.D., Murk, A.J., 2006. Thyroid Hormone mediated disruption of tadpole tail regression by hexabromocyclododecane (HBCD) and nona bromodiphenyl ether (BDE206). **Chemosphere in press**.

Shi, Y. B. 2000. Amphibian metamorphosis - from morphology to molecular biology: John Wiley & Sons. 288 p.

Smith, C. L. and O'Malley, B. W., 2004. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. **Endocr Rev 25**, 45-71.

Soderstrom, G., Sellstrom, U., de Wit, C. A., Tysklind, M., 2004. Photolytic debromination of decabromodiphenyl ether (BDE 209). **Environ Sci Technol 38**, 127-132.

Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N. S. F. O., 1995. The E-screen assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. **Environ Health Persp 103**, 113-122.

Tata, J. R., 1968. Early metamorphic competence of Xenopus larvae. **Dev Biol 18**, 415-440.

Tata, J. R., 1999. Amphibian metamorphosis as a model for studying the developmental actions of thyroid hormone. **Biochimie 81**, 359-366.

Thuresson, K., Bergman, A., Rothenbacher, K., Herrmann, T., Sjolin, S., Hagmar, L., Papke, O., Jakobsson, C., 2006. Polybrominated diphenyl ether exposure to electronics recycling workers – a **follow up study. Chemosphere 64, 1855-1861.**

Yamauchi, K., Ishihara, A., Fukazawa, H., Terao, Y., 2003. Competitive interactions of chlorinated phenol compounds with 3,3',5-triiodothyronine binding to transthyretin: detection of possible thyroid-disrupting chemicals in environmental waste water. **Toxicol Appl Pharm 187**, 110-117.

Yamauchi, K., Prapunpoj, P., Richardson, S. J., 2000. Effect of diethylstilbestrol on thyroid hormone binding to amphibian transthyretins. **Gen Comp Endocrinol 119**, 329-339.

Yaoita, Y. and Brown, D. D., 1990. A correlation of thyroid hormone gene expression with amphibian metamorphosis. **Gene Develop 4**, 1917-1924.

Yaoita, Y., Shi, Y., Brown, D. D., 1990. *Xenopus laevis* α and β Thyroid Hormone Receptors. **P Natl Acad Sci USA 87**, 7090-7094.

Zhang, J., Lazar, M. A., 2000. The mechanism of action of thyroid hormones. **Annu Rev Physiol 62**, 439-466.

6

Real-time automated measurement of *Xenopus laevis* tadpole behavior and behavioral responses following triphenyltin exposure using the Multispecies Freshwater Biomonitor (MFB)

Merijn Schriks, Melissa K. van Hoorn, Elisabeth J. Faassen, Joost W. van Dam, Albertinka J. Murk

Division of Toxicology, Wageningen University, Wageningen, The Netherlands

Aquatic Toxicology 77 (2006) 298-305

Abstract

The present study examines whether behavior of *Xenopus laevis* tadpoles, when measured with the Multispecies Freshwater Biomonitor (MFB), can be a sensitive and practical parameter for quantification of behavioral effects induced by toxic compounds. The MFB system is capable of automated simultaneous recording and integration of different types of movement over time. Basic tadpole behavior was studied under standard ambient temperature and colder conditions. At lower temperatures the time spent on low frequency behavior such as swimming and ventilation decreased, while higher frequency movements associated with subtle tail tip oscillations increased. Changes in behavior were also studied during the process of metamorphosis when both the morphology and physiology of tadpoles change. In the course of metamorphosis the tadpoles decreased the time spent on swimming and increased tail tip oscillations, especially in the period shortly before and during metamorphic climax. Additional experiments were performed to investigate whether the MFB could be used to quantify behavioral effects of exposure to a toxic compound. A 48 h exposure to a sub-lethal concentration of 1.25 $\mu g.L^{-1}$ triphenyltin (TPT) significantly increased low frequency behavior, whereas 5 $\mu g.L^{-1}$ TPT significantly reduced this type of behavior while the number of periods of total inactivity increased. One week after transferring the animals to clean water, registered behavior of tadpoles in the highest TPT group (5 $\mu g.L^{-1}$) was normal again for this developmental stage. The results show that the MFB can be used as a new tool for automated registration of sublethal toxic effects on tadpole behavior including recovery.

Introduction

Behavior of an organism can be considered as the integrated result of a diversity of biochemical and physiological processes (Walker et al., 2001). Although difficult to quantify, a single behavioral parameter can be more comprehensive than for example individual biochemical parameters. Many environmental pollutants affect various endocrine functions such as thyroid- and sex hormones and it is to be expected that they adversely affect related behavioral parameters. For example, experimentally altering thyroid hormone levels of juvenile mummichogs (*Fundulus heteroclitus*) by dosing water with triiodo-L-thyronine (T_3; active form of thyroid hormone) or PTU (6-n-propyl-2-thiouracil, a thyroid hormone antagonist) altered both spontaneous activity and prey capture rates (Carletta et al., 2000). Pollution-induced behavioral changes at environmental relevant concentrations have been found in a wide variety of vertebrate species (Relyea and Mills, 2001; Fraker and Smith, 2004). Such behavioral effects become ecologically relevant when they impair survival by reducing anti-predator responses (e.g. in fish and amphibians); (Madison et al., 1999; Belden et al., 2000; Chivers et al., 2001), growth (Wise et al., 1995), and reproduction (e.g. via impaired parental care) (Fry and Toone, 1981). At least one study has shown a direct correlation between reduced activity and predator-induced mortality for several species of tadpoles (Lawler, 1989). Although tadpoles have been proposed before to be good bioindicators of environmental pollution (Venturino et al., 2003), only 2.7 % of the literature in the last 25 years covers aspects of ecotoxicology concerning amphibians (Bridges, 2002). Basic knowledge on tadpole behavior and the influence of toxic compounds on tadpole behavior is even scarcer. To overcome the main difficulty associated with behavioral testing, namely acquisition of an objective, quantitative and a preferably non-laborious dataset, the last decades several automated procedures have been developed (Steele et al., 1985; Beitinger, 1990; Kotrschal and Essler 1995). One practical computer based automated approach employs the Multispecies Freshwater Biomonitor (MFB; Limco International, Ibbenbüren, Germany) (Gerhardt et al.,

1994; Gerhardt 1998a; Gerhardt 1998b; Gerhardt and Schmidt 2002). This technique is based on the detection of impedance changes across a test chamber due to movements of an organism in an alternating electrical field. It allows simultaneous recording of different types of movement of the organism in the test chamber. The MFB has been applied to several kinds of freshwater organisms, mainly to test behavioral effects of exposure to effluents of pharmaceutical and municipal wastewater on aquatic invertebrates and fish (Gerhardt, 2002). The objective of the present study was to study whether the MFB would be applicable to record and analyze tadpole behavioral responses to sub-lethal toxic exposures in an automated quantitative way. To establish the variety of natural occurring behavioral patterns of *Xenopus laevis* tadpoles and their characteristics when measured with the MFB, firstly basic behavior was studied both at room temperature (20 °C) and at a lower temperature (10 °C). Secondly, behavioral changes accompanying tadpole metamorphosis were studied because anurans undergo major morphological and physiological changes during the transition from tadpole to a juvenile frog (Shi, 2000). After establishing reference MFB values for basic tadpole behavior in this way, a behavioral experiment with a sub-lethal exposure to triphenyltin (TPT) was carried out. This compound has previously been shown to induce behavioral changes in tadpoles, and visual observations are therefore available (Semlitsch et al., 1995). TPT is one of the most commercially important organotin compounds in The Netherlands, and its major use is in fungicides to protect agricultural crops such as potatoes (Bock, 1981). TPT has been found in Zebra mussels (*Dreissena polymorpha*) collected from different parts of The Netherlands at concentrations ranging from 0.02 to 3.2 $\mu g.g^{-1}$ (dry wt.) (Staeb et al., 1995). Furthermore TPT has been detected in Dutch surface water and in precipitation at levels up to 0.7 and 76 $ng.L^{-1}$ respectively (Duyzer and Vonk, 2003). Finally, high TPT-levels have been reported (~3 $\mu g.L^{-1}$) in water samples that were collected from a national park in the USA (Jones-Lepp et al., 2004).

Animals, materials and methods

Animals

Fertilized *Xenopus laevis* eggs were obtained from the Netherlands Institute for Developmental Biology (NIOB, Utrecht, The Netherlands). Embryo's were raised in aerated tap water (further referred to as water) according to the standard conditions of the American Society for testing and materials (American Society for Testing and Materials, 1991). Briefly, in order to facilitate embryo development and increase hatching success, jelly coating of the eggs was removed by 2 min. incubation in L-cysteine (2 % in water, pH 8.1). Eggs were washed in water, after which unfertilized eggs were separated from fertilized eggs. Eggs were then maintained in water under a 12:12-h light:dark photoperiod. Animals were fed daily with approximately 30 mg food/tadpole/day as a suspension in water. The food consists of 500 g nettle powder (Jacob Hooy, Limmen, The Netherlands), 5 g BBL© yeast extract (Becton Dickinson Microbiology Systems, Cockeysville, USA), 5 g BBL© agar and 5 g coffee creamer (EDAH, Helmond, The Netherlands). When the larvae reached a size of 1-2 cm they were moved to a larger aquarium (20 L) and maintained at an approximate density of 50 animals per 10 L water (room temperature). Water was continuously aerated with an air-pump and 50 % refreshed every other day, but not during the weekends. All larvae were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975) (further abbreviated as NF) before use in the experiments.

Multispecies Freshwater Biomonitor

The principle of the MFB is described elsewhere (Gerhardt, 2002; Gerhardt and Schmidt 2002; Gerhardt et al., 2002; Bisthoven et al., 2004). It is capable of simultaneously recording movements at different frequencies associated with different types of behavior (20 recordings/sec, recording time 255 sec). Briefly, tadpoles were placed in a water-filled cylindrical test chamber made of plexiglass (∅ = 5.3 cm, height = 14.5 cm) between four stainless steel electrodes at the opposite chamber walls. One pair of electrodes produces an alternating current of

50 kHz over the chamber, whereas a second pair of non-current carrying electrodes measures impedance changes across the test chamber due to the animals' movements in the electric field. In order to prevent the tadpoles from escaping, both ends of the chambers were closed with nylon netting (mesh = 3 mm) covered screw rings. The chambers were submerged in an aquarium filled with tap water, so that the animals could swim freely in the chambers. During behavioral experiments the water was not aerated, in order to avoid interference of the MFB signal by air bubble generated vibrations. The photoperiod during all experiments was kept at a 12:12-h light:dark cycle. The raw data include intensity of movements expressed in volt (V) (further referred to as signal amplitude). Data analysis is based on a stepwise discrete Fast Fourier Transformation (FFT), thus generating a signal frequency histogram of the occurrence of frequencies between 0.5 and 8.5 Hz (in steps of 0.5 Hz) (Gerhardt, 1998b). The signals generated by the impedance generator were processed by a Microsoft Windows NT based PC using software supplied by the manufacturer (MFB, Version 2.1e; Limco International, Ibbenbüren, Germany). Background noise signals were measured in a chamber without a tadpole.

Establishing basic Xenopus laevis tadpole behavior

Four tadpoles (stage NF 53-54) per temperature were individually transferred to test chambers and placed in aquaria containing 4 L water of either 20 °C or 10 °C. Animals were allowed to acclimatize for 30 minutes, after which continuous MFB recording commenced in measuring periods of 255 seconds for the following hour. The experiment was carried out twice, and the data pooled. Typical behavior of *Xenopus laevis* tadpoles was visually monitored and afterwards compared with the frequency distribution histogram generated by the MFB. Special attention was paid to the three types of behavior that were distinguished by Gerhardt and co-workers (Gerhardt 2002) with Japanese Medaka (*Oryzias latipus*): swimming behavior, subtle fin and operculum movements (ventilation). In order to establish reference behavior of *Xenopus laevis* tadpoles during

metamorphosis, behavior of the tadpoles was recorded daily during 1.5 h in intervals of 255 seconds at room temperature (20 ± 2 °C) until completion of metamorphosis (25 days; stage NF 66). After each daily recording period, the animals were released from the test chambers, and fed with nettle powder as described earlier. By placing the test chambers vertically and leaving a bit of air in the top of the test chamber, the animals were able to breathe upon their metamorphosis from gill to lung breathers (Figure 1).

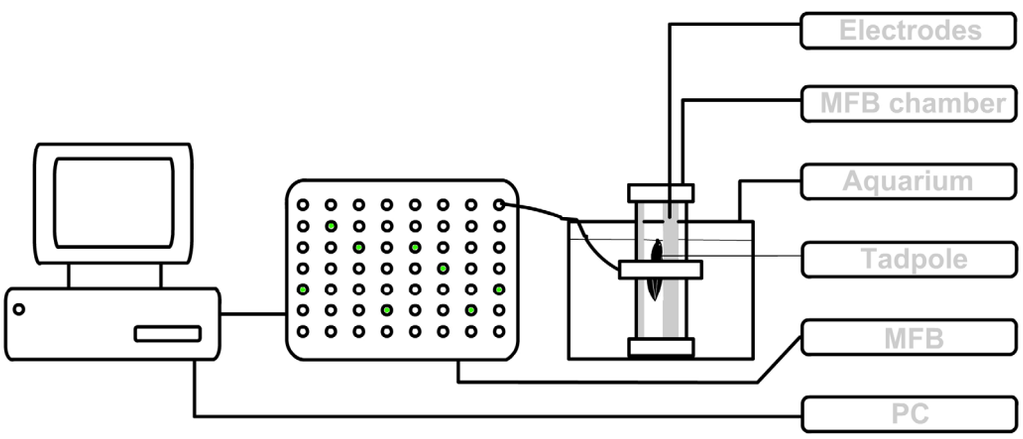


Figure 1. Illustration of the MFB test setup.

Behavioral responses after short-term exposure to triphenyltin (TPT)

The experiment was carried out as described (Semlitsch et al., 1995) with some modifications. Briefly, *Xenopus laevis* tadpoles (stage NF 50-51) were exposed for 48 h in a static design to 1.25 µg.L^{-1}, 2.5 µg.L^{-1} or 5 µg.L^{-1} triphenyltin chloride (TPT-cl; purity > 98 %; Riedel-de Haen, Seelze, Germany) in aerated tap water. Actual TPT exposure concentrations were not measured during this study, since TPT levels have been reported to be relatively stabile during a 48 h period. For example, TPT concentrations in a static design with fish were reported to decrease only after a 96 h (Tas et al., 1990). More recently, Fioramonti and co-workers showed stable TPT concentrations over a 72 h period without significant differences between nominal and measured TPT concentrations (Fioramonti et al., 1997). The 1.25 µg.L^{-1} and 2.5 µg.L^{-1} TPT concentrations are in the same range as concentrations found in the environment as reported (Jones-Lepp et al., 2005). The

5 µg.L^{-1} TPT was chosen because of reported behavioral effects on tadpoles but still is in the sub-lethal range. Two controls were included, namely water without or with the solvent control acetone (< 0.01 % v/v) (PA quality; Merck, Darmstadt, Germany). A short-term pilot exposure of *Xenopus laevis* tadpoles to TPT, revealed that exposure during 48 h to concentrations up to and including 5 µg.L^{-1} TPT are sub-lethal. A stock solution of 100 mg.L^{-1} TPT in acetone, kept at -18 °C to prevent degradation, was used throughout the experiment. On the day of exposure, 60 *Xenopus laevis* tadpoles were random distributed over five 2.5 L glass aquaria (12 per treatment), each containing 1 L of one of the five exposure concentrations and exposed for 48 h. During this period the animals were not fed, and water was not aerated to prevent loss of TPT as described (Tas et al., 1996). The pH was measured during the experiment and adapted with either 0.1 M NaOH or 0.1 M HCl to pH 7.4 when necessary. After the exposure period, tadpoles were placed in aquaria with 2.4 L fresh water and fed with nettle powder as described above. Tadpoles were held 24 h before behavioral testing commenced. At the start of each recording session, four tadpoles from each treatment group were randomly selected and individually assigned to twenty MFB test chambers (vertically submerged in water). After 30 minutes of acclimatization, behavior was recorded in three consecutive recording sessions during 1.5 h in measuring periods of 255 seconds, thus recording all 60 tadpoles. Thereafter the animals were transferred to clean aquaria with clean water (2.4 L), and fed with nettle powder. In order to investigate tadpole recovery from the TPT exposure, the behavior of four animals from each exposure group was measured again after keeping them one week in clean water.

Data analysis

Data were Fast Fourier Transformed (FFT) as described before (Gerhardt 1998b), which generated a histogram of signal frequencies occurring during the whole recording time of 255 seconds. Normality was tested with the Kolmogorov-Smirnov test and if transformation to a normal distribution failed, significance

between groups was tested with the nonparametric Mann-Whitney test for independent samples. After transferring MFB data to MS-Excel (Microsoft, Redmond, WA, USA) relative occurrence of frequencies were plotted against corresponding frequency bands. Differences between groups were analyzed with the Student's *t*-test or the Mann-Whitney test as a nonparametric alternative ($p \leq 0.05$ was considered as statistically significant). In some cases only the occurrence of activity in the 0.5 Hz band was plotted against time, since this frequency is representative for swimming behavior together with ventilation. Data of the TPT experiment were expressed relative to data of the water- and solvent control which were pooled.

Results

Basic behavior

Firstly the basic behavior of *Xenopus laevis* tadpoles under normal conditions at 20 °C was studied visually in combination with the MFB-signal. The characteristic plane of orientation of *Xenopus laevis* tadpoles in rest is head down at about 45°. The tadpoles do not use their entire tail for this behavior but only use the flagellum at the tip of their tail, which they rapidly oscillate while holding position (McDiarmid and Altig, 1999). *Xenopus laevis* tadpoles in stage NF 53-54 showed three types of characteristic behavior namely ventilation consisting of opening and closing of mouth/operculum, tail tip oscillation and swimming. Swimming resulted in an irregular, hetero-frequent, behavioral pattern (0.5-2.5 Hz, >1.3 V) with typically a high occurrence of low frequencies but not of high frequencies (Figure 2/top), whereas tail tip oscillation and ventilation generated a much more homo-frequent pattern with respectively a peak in the higher frequencies range (4-6.5 Hz, >1.3 V) (Figure 2/middle) and high occurrence of the low frequency band (0.5-2.5 Hz, <1.3 V) (Figure 2/bottom).

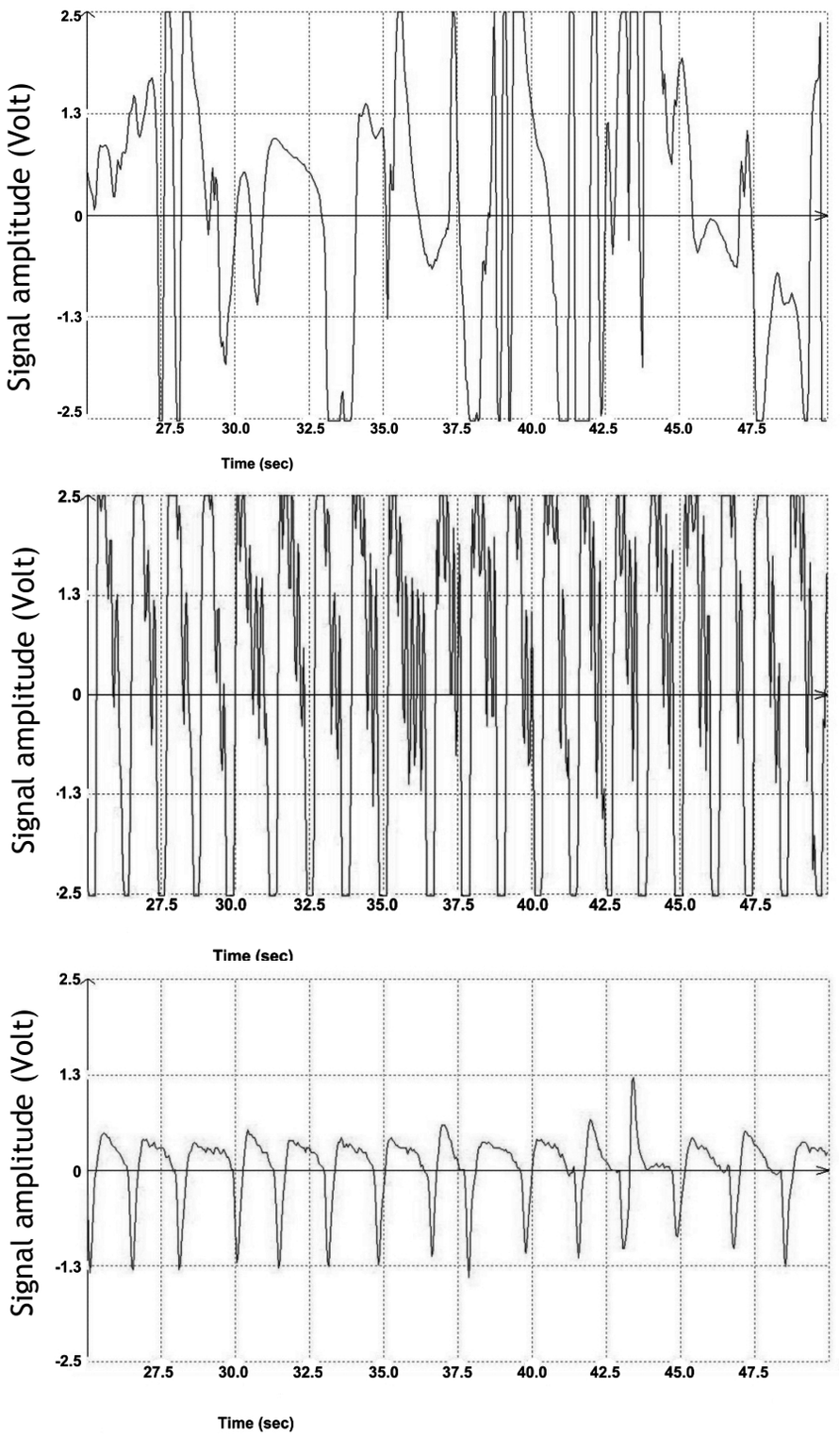


Figure 2. Characteristic behavioral pattern (raw data) in the Multispecies Freshwater Biomonitor (MFB) of *Xenopus laevis* tadpoles in Nieuwkoop and Faber (NF) stage 53-54 while (**top**) swimming; (**middle**) tail tip oscillation; and (**bottom**) ventilating. The voltage is a measure of the intensity of the movements, further referred to as signal amplitude. The figure illustrates 25 representative seconds of the total 255 second measuring interval.

Figure 3 shows the specific frequency distribution histograms of three types of behavior: swimming, ventilation and tail tip oscillation. There is a strong frequency overlap in the 0.5 Hz – 2.5 Hz region between swimming- and ventilation behavior. We therefore refer further to these types of behavior as low frequency behavior when there is no parallel visual observation. Tail tip oscillation, however, does not overlap with swimming or ventilation behavior.

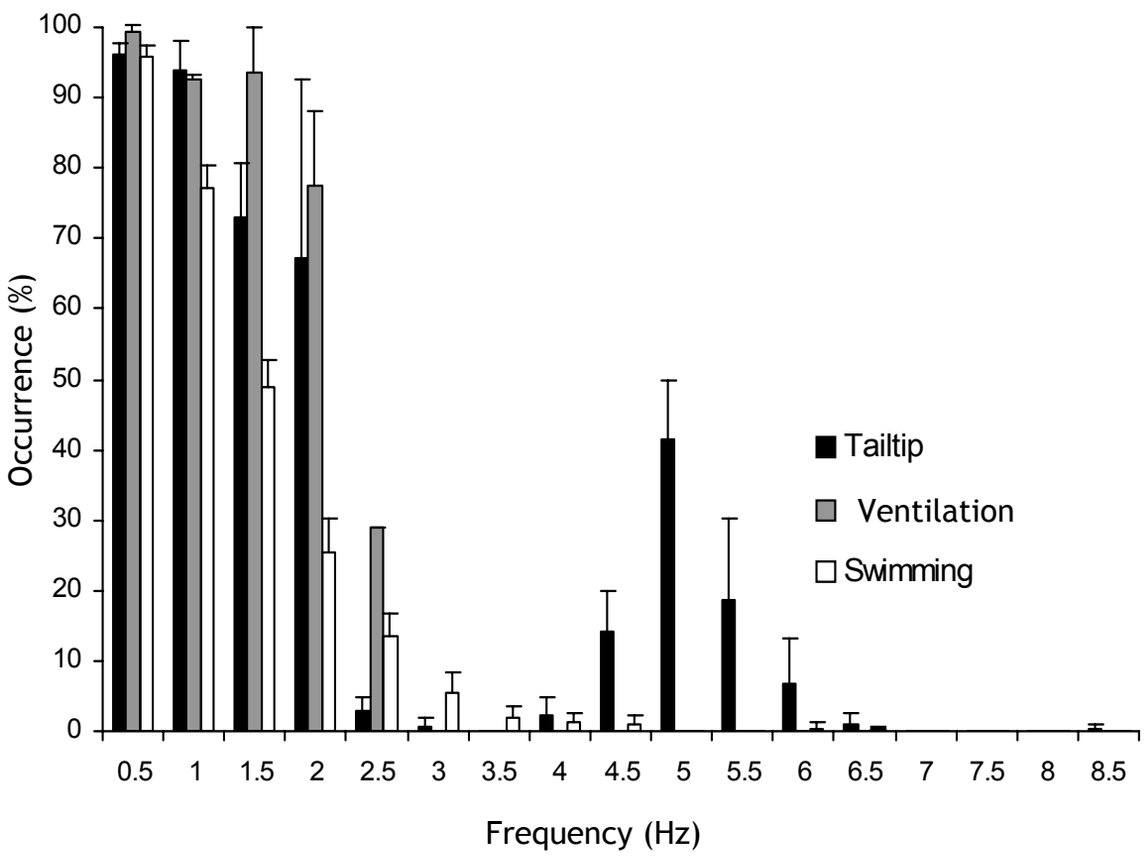


Figure 3. Frequency distribution histogram after fast Fourier transformation of behavioral signal amplitudes for the three specifically distinguished behaviours: swimming, ventilation and tail tip oscillation. Relative occurrence (as % of time moving in this frequency) of the frequencies 0.5 to 8.5 Hz is summarized in intervals of 0.5 Hz. Data are derived from eight acclimatised *Xenopus laevis* tadpoles in stage NF 53-54 at 20°C measured during 1 hour in intervals of 250 seconds.

Therefore especially at lower temperatures (10 °C), when the time spent on swimming-associated lower frequencies decreased, an increase up to 30 % of tail tip oscillations associated higher frequencies (5-5.5 Hz) occurred (Figure 4).

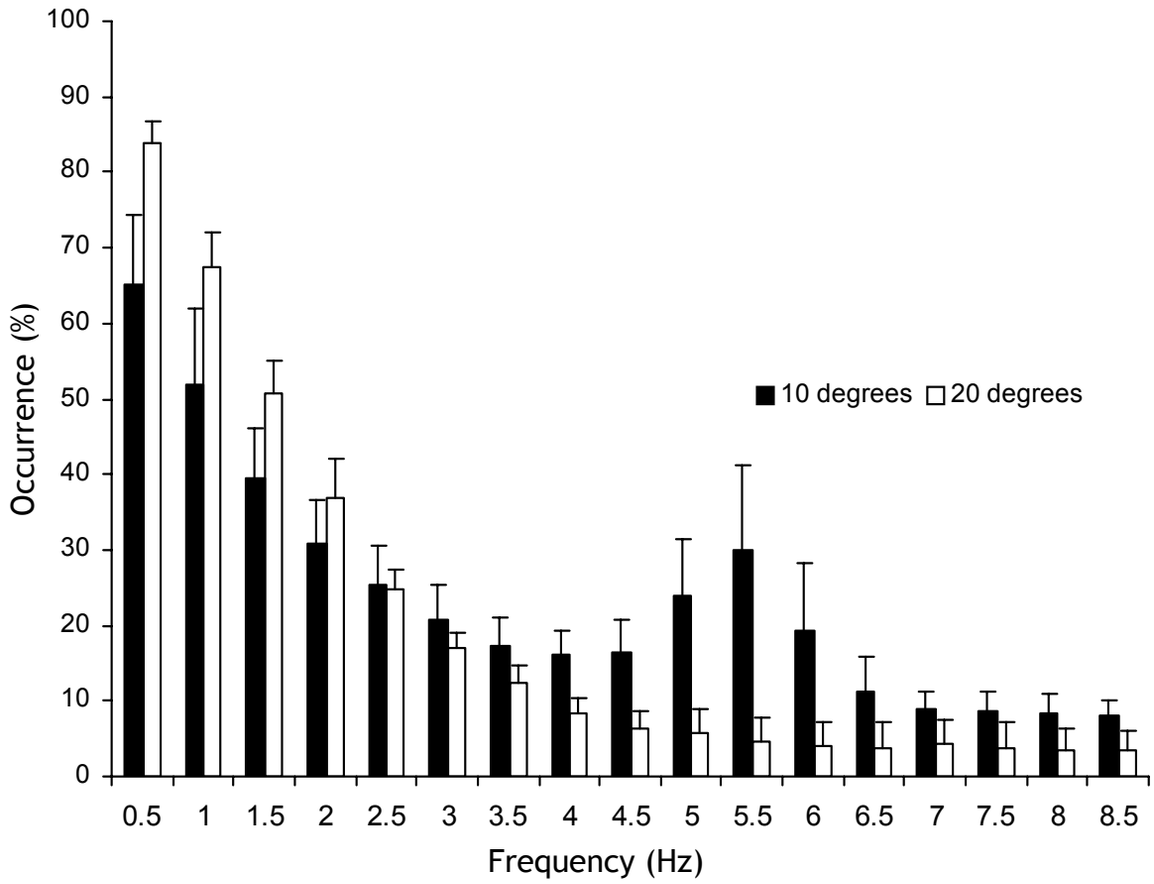


Figure 4. Frequency distribution histogram after fast Fourier transformation of the behavioral signal amplitudes of eight *Xenopus laevis* tadpoles in stage NF 53-54 at 10˚C and 20˚C. Tadpoles were acclimatised for 30 minutes to 10˚C or 20˚C and frequency distributions were measured during 1 hour in intervals of 255 seconds. Relative occurrence of the frequencies 0.5 to 8.5 Hz are summarized in intervals of 0.5 Hz as mean ± SD.

Metamorphosis

The behavioral pattern of *Xenopus laevis* tadpoles in the course of metamorphosis shows a slight increase of low frequency behavior (0.5 Hz, referring to swimming- and ventilation behavior) to 65 % until day 10 (stage NF 59) (Figure 5). Thereafter the time spent on low frequency behavior decreased steeply and remained below 19 % until the end of metamorphosis (except for day 12). No differences in specific high frequency tail tip oscillations were observed during this period.

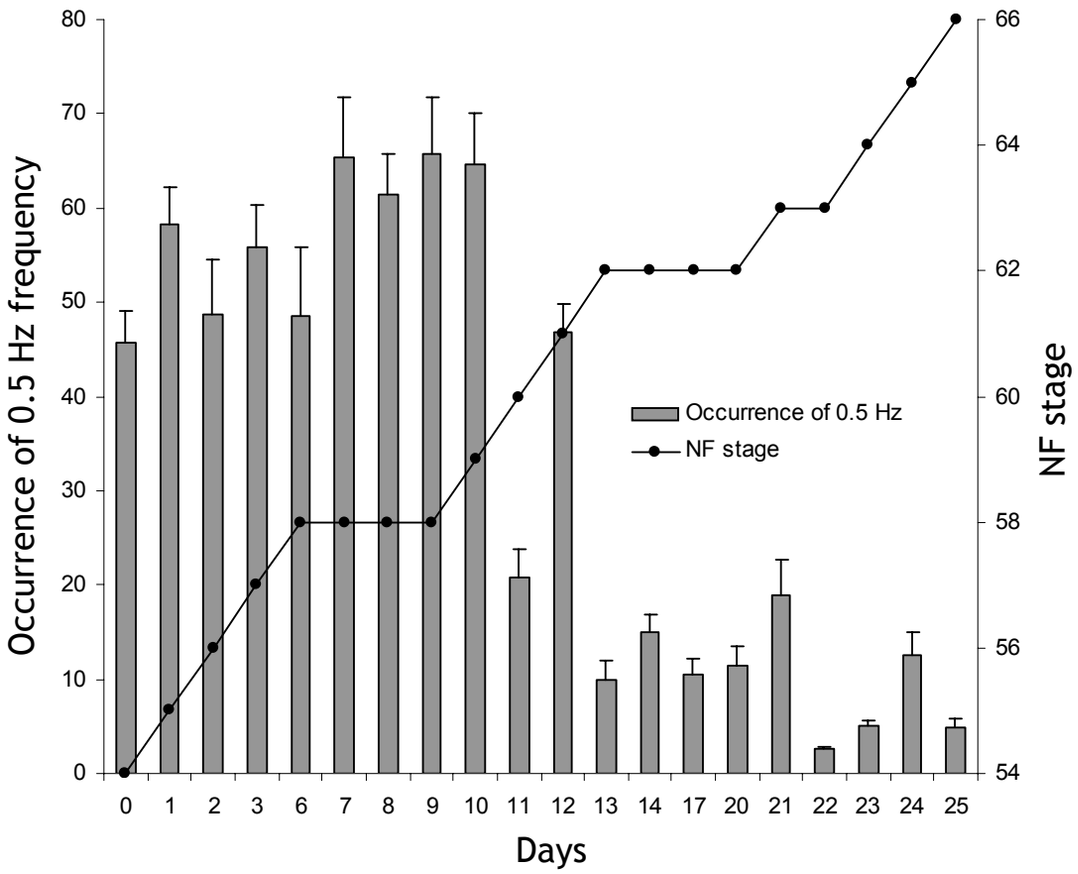


Figure 5. Frequency distribution histogram of low frequency behavior (swimming/ventilation) (as defined by the 0.5 Hz frequency) after fast Fourier transformation of behavioural signal amplitudes of one representative *Xenopus laevis* tadpole in the course of metamorphosis. Behavior was recorded daily in intervals of 255 seconds at room temperature (20 ± 2 °C). Relative occurrence of the 0.5 Hz frequency is calculated as mean ± standard error of mean (SEM), and developmental stages in the course of metamorphosis are derived from a representative tadpole.

Exposure to triphenyltin

After exposure to the highest TPT concentration (5 μg.L^{-1}), low (0.5 Hz) and high (5 Hz) frequency behavior of the tadpoles decreased significantly to less than 50 % of the acetone control ($p < 0.001$) (Figure 6a). No significant differences were observed between tadpoles in the acetone solvent control and the water control, and therefore these data were pooled. In the lowest TPT dosage-group (1.25 μg.L^{-}) the animals spend significantly (~24 %, $p \leq 0.005$) more time on low frequency behavior compared to the acetone control and significantly less (~60 %, $p < 0.01$) time on tail tip oscillation (see Table 1 for original data).

Table 1. Original data of relative occurrences (%) of different frequencies (0.5 Hz for swimming/ventilation and 5 Hz for tail tip oscillations) after 24 h exposure to triphenyltin, and after one week recovery in clean water.

		Relative occurrence (%) ± SEM of frequencies after exposure			Relative occurrence (%) ± SEM of frequencies after recovery	
Group	*n*	0.5 Hz	5 Hz	*n*	0.5 Hz	5 Hz
Solvent	24	48 ± 3	9 ± 1	8	54 ± 5	5 ± 0.6
1.25 μg.L^{-1}	12	60 ± 3	5 ± 1	4	68 ± 4	4 ± 0.5
2.5 μg.L^{-1}	12	48 ± 3	10± 1	4	51 ± 5	5 ± 1
5 μg.L^{-1}	12	23 ± 3	2 ± 0.5	2	45 ± 7	4 ± 0.9

Tadpoles exposed to 2.5 μg.L^{-1} TPT did not differ in behavior from animals in the acetone control. After allowing the tadpoles to recover in clean water for 1 week, behavior of tadpoles in all exposure groups, but low frequency behavior of tadpoles in the 1.25 μg.L^{-1} TPT exposure group, was restored to the level in the solvent control (Figure 6b). Interestingly, tadpoles in the highest exposure group (5 μg.L^{-1}) were more often completely inactive during measuring periods (255 seconds) than lower exposed animals (Figure 6c). This effect though, was not statistically significant due to the high standard deviation for this parameter. This trend is still apparent after maintaining animals one week on clean water. No mortality of tadpoles was observed during the exposure period, but 2 animals exposed to the highest TPT concentration (5 μg.L^{-1}) died during the recovery period in clean water.

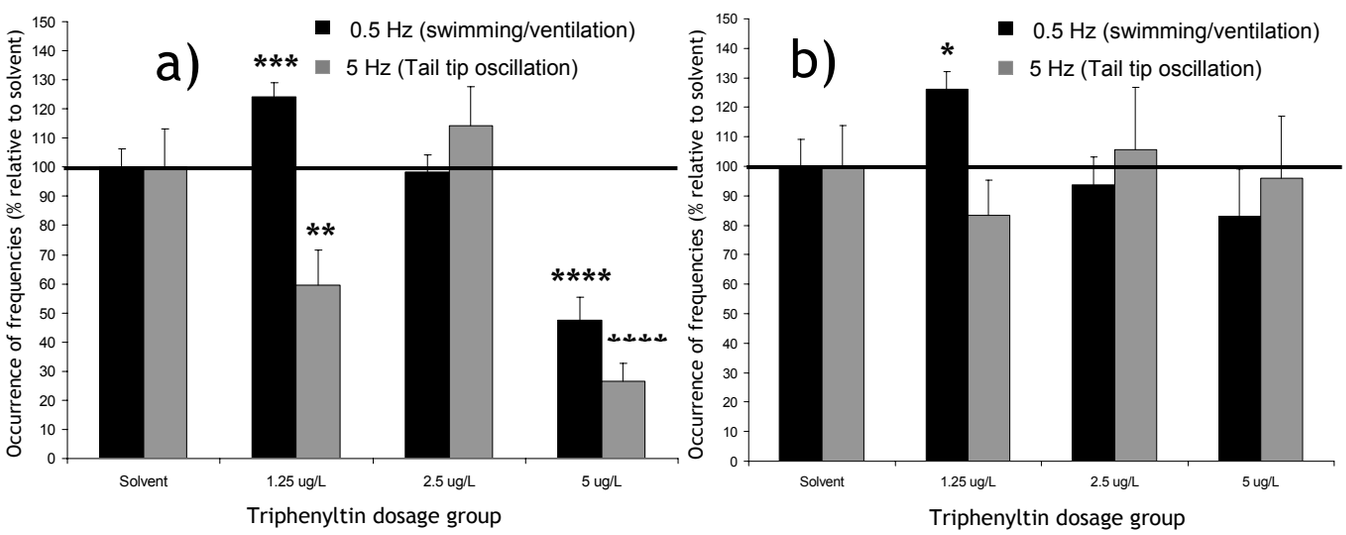


Figure 6. (see legend next page)

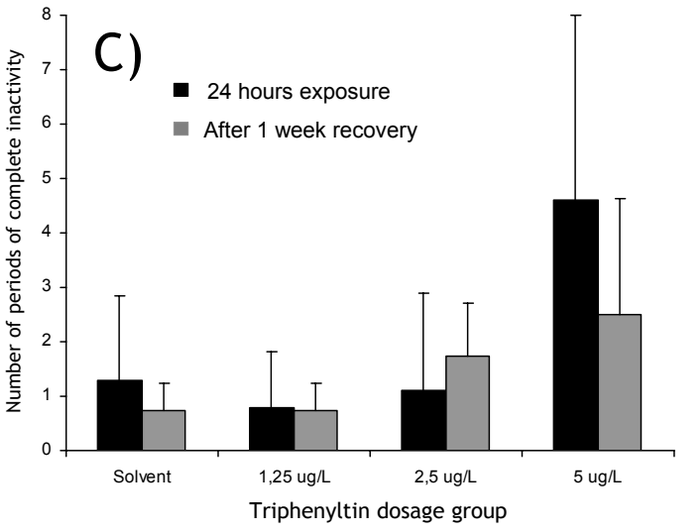


Figure 6. Frequency distribution histogram indicative of occurrence of tail tip oscillation (5 Hz) and swimming plus ventilation (0.5 Hz) per dosage group after **a**) 24 hours exposure of tadpoles (stage NF 50-51) to triphenyltin (n = 48) or solvent (n = 12) and **b**) after recovery for one week on clean water. Occurrence is presented as percentage relative to the acetone solvent control group. Error bars represent SEM. * Significantly different from solvent control ($p < 0.05$); ** $p < 0.01$; *** $p \leq 0.005$; **** $p < 0.001$. Figure **c** represents the number of times that tadpoles were completely inactive during 9 measuring periods of 255 seconds each.

Discussion

The present study aims at establishing basic behavioral patterns of *Xenopus laevis* tadpoles for toxicological studies with the Multispecies Freshwater Biomonitor (MFB) able of recording tadpole behavior in an automated and quantitative way. Up to now this technique has been mainly used to measure behavior of invertebrates and fish (Gerhardt et al., 1994). The basic behavior of *Xenopus laevis* tadpoles under normal conditions shows characteristic swimming movements with the tail at relatively low frequencies (0.5-2.5 Hz) with high signal amplitudes (> 1.3 V). To date the only literature available concerning MFB measurements and amphibian species (*Rana temporaria*) report on similar observations, with the exception that *R. temporaria* tadpoles take short resting brakes (Gerhardt et al., 1994). The frequency distribution histogram of *Xenopus laevis* tadpoles is comparable to that of Japanese medaka (*Oryzias latipes*) as reported in a study of

Gerhardt (Gerhardt, 2002). In both species swimming behavior results in a higher contribution of lower frequencies (0.5-2.5 Hz).

Ventilation of *Xenopus laevis* tadpoles was visible as a regular pattern with a frequency of 0.5-2.5 Hz, slightly lower than observed in *R. temporaria* (Gerhardt et al., 1994). Since ventilation and swimming behavior occur in the same frequency area, these types of behavior can only be separately distinguished if MFB measurements are carried out together with visual observations. This, however, is not the purpose of an automated system. Changes in behavior of the *Xenopus laevis* tadpoles were observed with the MFB when the animals were cooled to 10 °C. As expected a decrease in swimming and ventilation associated low frequency behavior occurred, accompanied by an increase in high frequency behavior at 5.5 Hz (associated with tail tip oscillation). Development in *Xenopus laevis* tadpole basic behavior was registered with the MFB starting with animals in developmental stage NF 54 until completion of metamorphosis (stage NF 66). The basic behavior of the animals was fairly constant until stage NF 58, just before the peak of metamorphic changes. From stage NF 59 on low frequency behavior associated with swimming is seriously reduced (Figure 5). Comparable behavioral changes during metamorphosis have been shown before with manual observations (Hourdrey et al., 1996). Our observations are in accordance with findings that the swimming performance of tadpoles is poor during the peak of metamorphic transition (Wassersug and Sperry, 1977), making them more vulnerable for predation (Arnold and Wassersug, 1978). Because of the observed shift in basic behavior in the course of metamorphosis it is advised to use *Xenopus laevis* tadpoles in developmental stages NF 54-58 for behavioral toxicological experiments. Moreover, this study shows that behavioral patterns of these animals in premetamorphic stages are relatively constant. As a proof of principle an earlier toxicological relevant behavioral experiment with triphenyltin exposed tadpoles (Semlitsch et al., 1995) was now performed with the MFB. Exposure of *Xenopus laevis* tadpoles to the highest concentration TPT (5 $\mu g.L^{-1}$) reduced both low (0.5 Hz) and high (5 Hz) frequency behavior, which is associated with

swimming/ventilation and tail tip oscillation respectively (Figure 6a). In the same group the average periods of total inactivity increased 3.5-fold relative to solvent control (Figure 6c) which can be considered as abnormal behavior for tadpoles. Although this dose-related effect was not statistically significant, it suggests that the condition of the animals is seriously affected. In the field this could negatively influence tadpole survival when predator avoidance and foraging behavior are affected. Our results are in agreement with earlier work carried out by Semlitsch and co-workers (Semlitsch et al., 1995), who observed a decrease in swimming behavior of *Rana esculenta* tadpoles exposed during 24 hrs to concentrations up to 20 $\mu g.L^{-1}$ TPT. This concentration, however, appeared to be lethal for *Xenopus laevis* tadpoles because in a pilot experiment we found that a TPT concentration of 10 $\mu g.L^{-1}$ caused 100 % mortality within 24 h. It is not yet known whether the lower TPT tolerance observed in *Xenopus laevis* tadpoles is due to species or life-stage differences or to differences in test conditions influencing e.g. the bioavailability of TPT. Differences in sensitivity for environmental pollutants, such as sodium nitrate, between various species of tadpoles have been reported before (Schuytema and Nebeker, 1999). Interestingly, an increase in swimming / ventilation behavior was observed in the lowest TPT group (1.25 $\mu g.L^{-1}$), with a corresponding decrease of behavior associated with tail tip oscillations, but not with a decrease in periods of inactivity. An increase in (feeding) behavior was observed by Semlitsch and co-workers (Semlitsch et al., 1995), and they explained this as a possible result of hormesis. However, the difference between the lethal (> 5 $\mu g.L^{-1}$) and swimming stimulating concentration in our experiment is only 4-fold, which is very small for a hormesis effect. An alterative hypothesis is that increased low frequency behavior in the lower concentration may be explained by an avoidance response to organotin exposure which has earlier been described for different species, such as fish (Pinkney et al., 1985). After keeping the animals on clean water for one week, the activity of the tadpoles in the highest TPT group (5 $\mu g.L^{-1}$) appear normal again although this data was based on a low number of animals. Interestingly, an increased activity level (0.5 Hz) in the lowest TPT group

(1.25 μg.L^{-1}) was maintained, but tail tip oscillations (5 Hz) restored to the level of animals in the solvent control. It remains to be investigated which mechanism underlies the observed behavioral effects, and cannot be completely explained at the moment. At least partially due to high variation in manually registered swimming behavior, Semlitisch and co-workers were not able to reveal significant effects between tadpoles exposed to 5 μg.L^{-1} TPT and the vehicle control. In the present study however, significant effects were obtained for these concentrations with a much less laborious method, which is not subject to inter-observer variance.

Conclusion

The present study successfully applies the Multispecies Freshwater Biomonitor (MFB) to study behavioral changes of *Xenopus laevis* tadpoles. Changes in behavior were recorded in an automated way during development, under different ambient conditions and after acute exposure to sublethal concentrations of triphenyltin (TPT). The effects in the highest concentration of 5 μg.L^{-1} TPT resulted in significantly reduced swimming behavior and tail tip oscillation while the number of periods of total inactivity increased. In the lowest concentration (1.25 μg.L^{-1} TPT) swimming activity significantly increased. After a full week on clean water the behavior of the animals formerly exposed to the highest concentration appeared back to control values again. Overall, the results show that the MFB can be used as a new tool for automated registration of sublethal toxic effects on tadpole behavior.

Acknowledgements

The helpful comments and critical discussions on the manuscript as provided by prof. Ivonne M.C.M. Rietjens (Wageningen University, Toxicology section, The Netherlands) are gratefully acknowledged. Kees Koster of the Netherlands Institute for Developmental Biology (NIOB) is thanked for provision of fertilized *Xenopus laevis* eggs. This research was partly financially supported by a CEFIC-EMSG grant (project coordinator Christ de Rooij).

References

American Society for Testing and Materials, 1991. Standard guide for conducting the frog embryo teratogenesis assay-Xenopus. Annual book of ASTM standards. p 1-11.

Arnold, S. J., Wassersug, R. J., 1978. Differential predation on metamorphic anurans by garter snakers (*Thamnophis*): social behavior as a possible defense. Ecology 59, 1014-1022.

Beitinger, T. L., 1990. Behavioral reactions for the assessment of stress in fishes. **J Great Lakes Res 16**, 495-528.

Belden, L. K., Wildy, E. L., Hatch, A. C., Blaustein, A. R., 2000. Juvenile western toads, *Bufo boreas*, avoid chemical cues of snakes fed juvenile, but not larval, conspecifics. **Anim Behav 59**, 871-875.

Bisthoven de, L. J., Gerhardt, A., Soares, A., 2004. Effects of acid mine drainage on larval Chironomus (*diptera, chironomidae*) measured with the multispecies freshwater biomonitor. **Environ Toxicol Chem 23**, 1123-1128.

Bock, R., 1981. Triphenyltin compounds and their degradation products. **Residue Rev 79**, 1-270.

Bridges, C. M., 2002. Tadpoles balance foraging and predator avoidance: Effects of predation, pond drying, and hunger. **J Herpetol 36**, 627-634.

Carletta, M., Weis, J. S., Weis, P., 2000. Response to thyroid hormone in juvenile mummichogs (*Fundulus heteroclitus*) from a polluted vs. a reference site. Paper presented at the conference on Endocrine Disruptors in the Marine Environment: Impacts on Marine Wildlife and Human Health. Jackson Laboratory, ME (USA).

Chivers, D. P., Wildy, E. L., Kiesecker, J. M., Blaustein, A. R., 2001. Avoidance response of juvenile Pacific treefrogs to chemical cues of introduced predatory bullfrogs. **J Chem Ecol 27**, 1667-1676.

Duyzer, J.H. and Vonk, A.W., 2003. Atmospheric deposition of pesticides, PAHs and PCBs in The Netherlands. Apeldoorn: TNO, The Netherlands. 105 p.

Fioramonti, E., Semlitsch, R. D., Reyer, H. U., Fent, K., 1997. Effects of triphenyltin and pH an the growth and development of *Rana lessonae* and *Rana esculenta* tadpoles. **Environ Toxicol Chem 16**, 1940-1947.

Fraker, S. L., Smith, G. R., 2004. Direct and interactive effects of ecologically relevant concentrations of organic wastewater contaminants on *Rana pipiens* tadpoles. **Environ Toxicol 19**, 250-256.

Fry, D. M., Toone, C. K., 1981. DDT-induced feminization of gull embryos. **Science 213**, 922-4.

Gerhardt, A., Svensson, E., Clostermann, M., Fridlund, B., 1994. Monitoring of behavioral patterns of aquatic organisms with an impedance conversion technique. **Environ Int 20**, 209-219.

Gerhardt, A., 1998a. New online biomonitoring System for *gammarus pulex* (L.) (*crustacea*): In situ test below a copper effluent in south Sweden. **Environ Sci Technol 32**, 150-156.

Gerhardt, A., 1998b. Whole Effluent Toxicity Testing with *Oncorhynchus mykiss* (Walbaum 1792): Survival and Behavioral Responses to a Dilution Series of a Mining Effluent in South Africa. Arch **Environ Contam Toxicol 35**, 309-316.

Gerhardt, A., 2002. Short-term responses of *Oryzias latipes* (Pisces: Adrianichthyidae) and Macrobrachium nipponense (*Crustacea: Palaemonidae*) to municipal and pharmaceutical waste water in Beijing, China: survival, behaviour, biochemical biomarkers. **Chemosphere 47**, 35-47.

Gerhardt, A., Schmidt, S., 2002. The Multispecies Freshwater Biomonitor - A potential new tool for sediment biotests and biomonitoring. **J Soils Sediments May**, 1-4.

Gerhardt, A., Schmidt, S., Hoss, S., 2002. Measurement of movement patterns of *Caenorhabditis elegans* (*Nematoda*) with the Multispecies Freshwater Biomonitor(R) (MFB) - a potential new method to study a behavioral toxicity parameter of nematodes in sediments. **Environ Pollut 120**, 513-516.

Hourdrey, J., L'Hermite, A., Ferrand, R., 1996. Changes in the digestive tract and feeding behavior of anuran amphibians during metamorphosis. **Physiol Zool 69**, 219-251.

Jones-Lepp, T. L., Varner, K. E., Heggem, D., 2004. Monitoring dibutyltin and triphenyltin in fresh waters and fish on the United States using micro-liquid chromotography-electrospray/ion trap mass spectrometry. **Arch Environ Contam Toxicol 46**, 90-95.

Kotrschal, K., Essler, H., 1995. Goals and approaches in the analysis of locomotion in fish, with a focus on laboratory studies. **Selective Rev Fish Sci 3**, 171-200.

Lawler, S. P., 1989. Behavioural responses to predators and predation risk in four species of larval anurans. **Anim Behav 38**, 1039 - 1047.

Madison, D. M., Maerz, J. C., McDarby, J. H., 1999. Optimization of predator avoidance by salamanders using chemical cues: Diet and diel effects. **Ethology 105**, 1073-1086.

McDiarmid, R. W., Altig, R., 1999. Tadpoles - the biology of anuran larvae. London: The University of Chicage Press, Ltd.

Nieuwkoop, P. D. and Faber, J., 1975. Normal table of *Xenopus laevis*. Amsterdam, The Netherlands: North Holland Publishing.

Pinkney, A. E., Hall, L. W., Lenkevich, M. J., Burton, D. T., Zeger, S., 1985. Comparison of Avoidance Responses of an Estuarine Fish, *Fundulus heteroclitus*, and Crustacean, *Palaemonetes pugio*, to Bis (Tri-N-Butyltin) Oxide. **Water Air Soil Pollut 25**, 33-40.

Relyea, R. A., Mills, N., 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). **Proc Natl Acad Sci 98**, 2491-2496.

Schuytema, G. S., Nebeker, A. V., 1999. Comparative effects of ammonium and nitrate compounds on Pacific treefrog and African clawed frog embryos. **Arch Environ Contam Toxicol 36**, 200-206.

Semlitsch, R. D., Foglia, M., Mueller, A., Steiner, I., Fioramonti, E., Fent, K., 1995. Short-Term Exposure to Triphenyltin Affects the Swimming and Feeding-Behavior of Tadpoles. **Environ Toxicol Chem 14**, 1419-1423.

Shi, Y. B., 2000. Amphibian metamorphosis - from morphology to molecular biology: John Wiley & Sons. 288 p.

Staeb, J. A., Frenay, M., Freriks, I. L., Brinkman, U. A. Th., Cofino, W. P., 1995. Survey of nine organotin compounds in the Netherlands using the zebra mussel (*Dreissena polymorpha*) as a biomonitor. **Environ Toxicol Chem 14**, 2023-2032.

Steele, C. W., Owens, D. W., Scarfe, A. D., Thomas, P., 1985. Behavioral assessment of the sublethal effects of aquatic pollutants. **Mar Pollut Bull 16**, 221-224.

Tas, J. W., Opperhuizen, A., Seinen, W., 1990. Uptake and elimination kinetics of triphenyltin hydroxide by two fish species. **Toxicol Environ Chem 28**, 129–141.

Tas, J. W., Keizer, A., Opperhuizen, A., 1996. Bioaccumulation and lethal body burden of four triorganotin compounds. **Bull Environ Contam Toxicol 57**, 146-54.

Venturino, A., Rosenbaum, E., De Castro, A. C., Anguiano, O. L., Gauna, L., De Schroeder, T. F., De D'Angelo, A. M. P., 2003. Biomarkers of effect in toads and frogs. **Biomarkers 8**, 167-186.

Walker, C. H., Hopkin, S. P., Sibly, R.M., Peakall, D.B., 2001. Principles of Ecotoxicology. London, UK: Taylor & Francis.

Wassersug, R. J., Sperry, D. G., 1977. The relationship of locomotion to differential predation on Pseudacris triseriata (*Anura: Hylidae*). **Ecology 58**, 830-839.

Wise, L. D., Gordon, L. R., Soper, K. A., Duchai, D. M., Morrissey, R. E., 1995. Developmental neurotoxicity evaluation of acrylamide in Sprague-Dawley rats. **Neurotoxicol Teratol 17**, 189-198.

7

Summary and general discussion

The last years, both scientific and public concern about the possible threat of compounds in the environment that may affect endocrine functions has increased. Thus far, the majority of endocrine disruptor research has focused on the interference of compounds with the sex hormone homeostasis. Less attention has been paid to disruption of the hypothalamus-pituitary-thyroid (HPT) axis, despite the fact that several lines of evidence suggest that this system is also susceptible to disruption by compounds present in the environment (Brouwer et al., 1998). Disturbances in thyroid hormone (TH) homeostasis may lead to mosaic effects on development, growth patterns and metabolism in vertebrates including mammals and amphibians (chapter 1.4). Recent studies now indicate that the Thyroid hormone Receptors (TRs) also are targets of industrial compounds (Zoeller et al., 2005), but the effects and mechanisms are difficult to establish since suitable test assays are very limited. In part, this may be because research has generally focused on the ability of compounds to affect TH transport, TH metabolism and TH blood plasma levels but not on disruption of TR mediated TH-action.

The research presented in this thesis aims to enhance insight into the mechanisms underlying the effects of endocrine disrupting compounds on TR-mediated TH-action. To this end, newly developed and validated *in vitro* and *in vivo* assays are applied in addition to an *ex vivo* assay using isolated tail tissue. This *ex vivo* model more resembles the natural situation for cells than *in vitro* assays, but excludes the feedback mechanisms from the TH axis or extracellular metabolism which may obscure TR-mediated responses. In the assays exposure is performed in combination with TH in order to closer approach the exposure of cells under physiological conditions, where TH is present during important vertebrate fetal developmental periods. The *in vitro*, *ex vivo* and *in vivo* assay responses also are compared to study to what degree the *in vitro* and *ex vivo* assays can predict the *in vivo* thyroid hormone disrupting potency of compounds.

7.1 Summary of results

Several *in vitro*, *ex vivo* and *in vivo* assays have been applied within the scope of this thesis, the results of which are described in the section below.

In vitro assays

The T-screen employs a rat pituitary (GH_3) cell line, the cell proliferation of which is dependent on the presence of the active thyroid hormone 3,3',5-triiodo-L-thyronine (T_3) and mediated by the thyroid hormone receptors (TRs) (chapter 2). Agonistic, antagonistic and potentiating compounds, including brominated diphenylethers (BDEs) and other halogenated compounds, were studied with or without co-incubation with T_3 at its EC_{50} (0.25 nM) (chapter 2). A specifically synthesized agonist, the T_3-like hydroxylated BDE (further referred to as T_3-like BDE-OH) was roughly 1000 times less potent than T_3, whereas the T_4-like BDE-OH was roughly 5500 times less potent. The T_2-like BDE-OH did not induce TR-mediated responses in the T-screen. The model T_3 antagonist amiodarone antagonized T_3-action with an IC_{50} of 2.1 μM. Of the brominated flame retardants (BFRs), BDE206 acted as a potent antagonist with an IC_{50} of 449 nM. The model T_3 potentiator diiodobiphenyl (DIB) potentiated T_3-action with 15 % (at 10 μM) relative to T_3 at its EC_{50} (Schriks, unpublished results). Another compound that was found to be a potentiator was hexabromocyclododecane (HBCD), which stimulated T_3-induced cell proliferation up to 176 % (at 1 μM) relative to the T_3 at its EC_{50}, but was inactive when dosed without T_3 (chapter 2). Such potentiation was later also found for BDE28 which stimulated the T_3 response at its EC_{50} up to 66 % (at 1 μM) but did not act as a TR agonist itself (Hamers et al., 2006).

Since TRs occur in different isoforms (α and β) and play important roles in the tissue specific effects of T_3, the TR isoform selectivity of compounds with an effect in the T-screen was further investigated (chapter 3). Since T_3 is present under physiological conditions during important fetal vertebrate developmental stages *in vivo*, the selected compounds were tested in combination with T_3 at its EC_{50}. For this purpose the green monkey kidney fibroblast (CV-1) cell line was transiently

transfected with either Xenopus TRα or TRβ and a luciferase reporter gene as previously described for testing of pharmaceutical compounds (Furlow et al., 2004). The T_3-like BDE-OH, which was an agonist in the T-screen, stimulated T_3-induced TRα activation, but not T_3-induced TRβ activation. The antagonist BDE206 did antagonize both T_3-induced TRα and TRβ activation. DIB and BDE28, shown to potentiate T_3-action in the T-screen (Schriks et al., 2006 (chapter 2); Hamers et al., 2006), acted via TRα and TRβ, respectively. A concentration of 100 nM BDE28 almost tripled T_3-induced TRβ activation. Interestingly, the potentiating effect of HBCD which occurred in the T-screen (chapter 2) was not observed in the CV-1 cells. This suggests that the potentiation of T_3-action observed for HBCD in chapter 2, 4 and 5, occurs via different mechanisms than for DIB and BDE28 which more closely resemble the molecular structure of T_3.

Ex vivo assay

A method to demonstrate the effects of compounds on TR-mediated T_3-action in a cell system closer to the *in vivo* situation, but without physiological hormonal feed-back mechanisms was based on TH-mediated amphibian metamorphosis (Weber et al., 1967; Furlow et al., 2004) (chapter 4). Isolated tadpole tail tips exposed to T_3 in organ culture (*ex vivo*) undergo specific regression in a dose-dependent fashion. BDE206 significantly antagonized T_3-induced tail tip regression at a concentration of 100 nM or higher. HBCD potentiated T_3-induced tail tip regression at 1000 nM or higher, but did not have any effect when dosed alone. The results obtained with the tail tip exposures are in accordance with T-screen effects as quantified in chapter 2, and occurred at compound concentrations that were only 5 – 50 times those of T_3.

In vivo assays

To examine whether HBCD and BDE206 elicit their effects on T_3-action *in vivo*, an *in vivo* proliferation bioassay was applied using one-week old *Xenopus laevis* tadpoles (Schreiber et al., 2001) (chapter 5). T_3-induced cell proliferation in

tadpole tissues was quantified with a fluorescent antibody against the phospho-histone H3 mitosis marker and image analysis. Cell proliferation in brain and the rostral head region responded to T_3 in a dose-dependent fashion (EC_{50} brain = 0.77 nM; EC_{50} rostral head region = 0.57 nM), with the concentration dependent profile in brain being less steep and therefore chosen for further *in vivo* testing of compounds. HBCD potentiated T_3-induced cell proliferation in the brain, whereas BDE206 appeared to be inactive. To investigate this discrepancy, the effect of BDE206 was also quantified in the rostral head region which revealed that BDE206 acts as an antagonist in this tissue (chapter 5). This tissue-specific difference could be related to differential tissue expression of TR isoforms, or due to toxicokinetic differences of the structurally very different BDE206 and HBCD.

Finally, the effect of the T_3-antagonist triphenyltin (TPT) (Gutleb et al., 2005) was studied on a functional endpoint, namely behavioral changes of *Xenopus laevis* tadpoles (chapter 6). To this end, the Multispecies Freshwater Biomonitor (MFB) was adapted for quantifying basic tadpole behavior. Firstly, behavior was quantified at two different temperatures (10 °C and 20 °C) and secondly throughout the course of metamorphosis. The MFB proved to be capable of automated simultaneous recording and integration of different types of movements over time. For further validation of the method a 48 h exposure of tadpoles to sublethal concentrations of TPT was performed followed by a recovery period. Significantly increased low frequency behavior was observed with 1.25 $\mu g.L^{-1}$ TPT, whereas 5 $\mu g.L^{-1}$ TPT significantly reduced this type of behavior and increased the number of periods of total inactivity. One week after transferring the animals to clean water, registered behavior of tadpoles in the highest TPT group (5 $\mu g.L^{-1}$) was normal again for this developmental stage. The results reveal that the MFB can be used as a new tool for automated registration of sublethal toxic effects on tadpole behavior, including recovery.

In general, it can be concluded that the assays applied in this thesis specifically respond to the action of T_3. In the next paragraph the responses of the assays to the action of T_3 are described in relation to their specific characteristics.

7.2 Relative responses of assays to T_3

The T-screen (chapter 2), TRα/β specific reporter gene assays (chapter 3), *ex vivo* tail tip assay (chapter 4) and *in vivo* proliferation assay (chapter 5), demonstrate a dose-response related effect following exposure to T_3 (Figure 1).

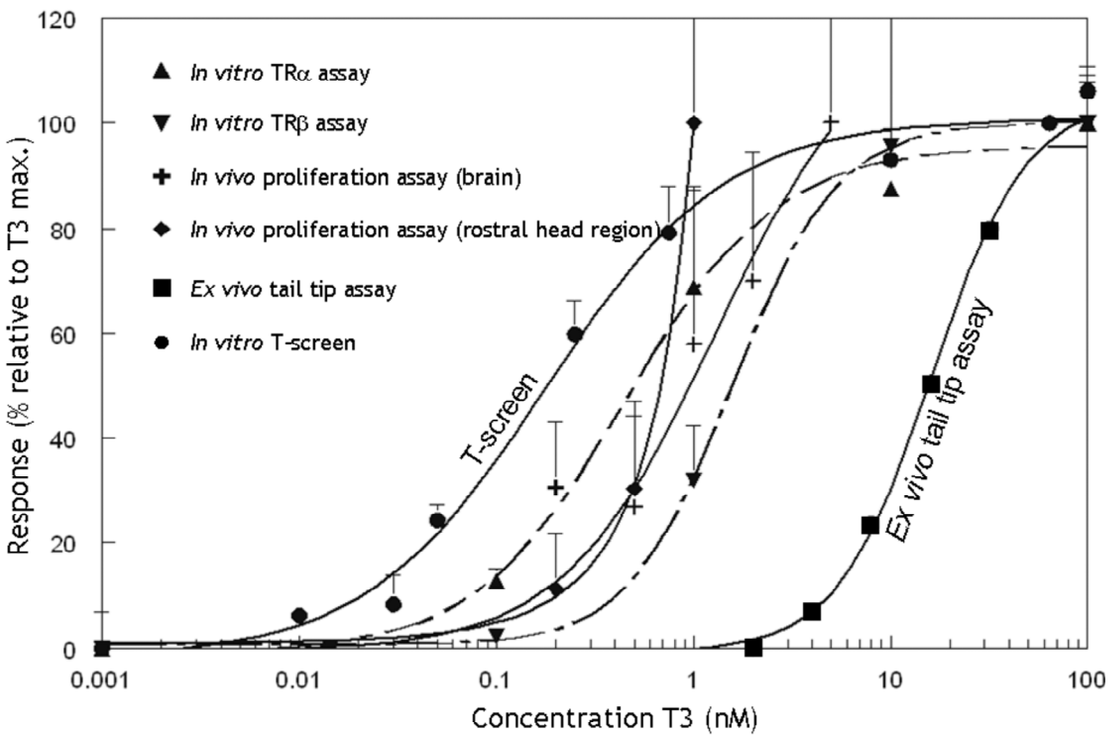


Figure 1. Dose response curves for different bioassays used in this thesis. Symbols represent: ● *In vitro* T-screen (chapter 2); ▲ *In vitro* TRα reporter gene assay (chapter 3); ▼ *in vitro* TRβ reporter gene assay (chapter 3); + *In vivo* proliferation assay (brain) (chapter 5); ♦ *In vivo* proliferation assay (rostral head region) (chapter 5); ■ *Ex vivo* tail assay (chapter 4) (dose-response curve derived from data as presented earlier; Furlow et al., 2004). Responses have been calculated relative to the maximum T_3-response which was set at 100 %.

The sensitivity to T_3 varies with the bioassay used. In the *ex vivo* tail tip assay, the EC_{50} of T_3 was 10-80 times higher than in the other bioassays (table 1). There may be several reasons for this difference in sensitivity. In the *ex vivo* tail tip assay, actual target cell exposure will likely be much lower than in the other assays, due to the more complex structure of multi-layered tadpole tail tissue and the absence of a blood circulation to actively transport the compounds to the target cells. The most sensitive bioassay was the T-screen, with an EC_{50} for T_3 that was 2-7 times

lower than the EC_{50} obtained in the TRα/β specific reporter gene assays. This higher sensitivity could be due to the longer exposure time in the T-screen which is 96 h compared to 48 h in the TRα/β specific reporter gene assays. Interestingly, the *in vivo* cell proliferation assay is only slightly less sensitive to T_3 than the *in vitro* T-screen. This may partially be due to effective uptake and distribution of T_3 by the small one-week old tadpoles and the high sensitivity of tadpole tissue to T_3-action. The brain of tadpoles, however, was slightly less sensitive to T_3 than the rostral head region. This may be best explained by a limited capacity for T_3 to efficiently pass the blood brain barrier.

Table 1. Relative sensitivities of the assays to T_3 exposure via the assay medium.

Assay	Exposure period (d)	EC_{50} (T_3) nM
In vitro T-screen	3	0.21
In vitro TRα reporter gene assay	2	0.46
In vitro TRβ reporter gene assay	2	1.5
In vivo proliferation assay (brain)	6-8	0.77
In vivo proliferation assay (rostral head region)	6-8	0.55
Ex vivo tail tip assay	6	16

In the next paragraph the activities of the tested compounds in the various assays are discussed in relation to compound-characteristics and expected mechanisms of action (potentiating, agonizing, antagonizing).

7.3 Possible mechanisms for thyroid hormone disruption based on the results of this thesis

The results described in this thesis show that three types of disruption of TH-mediated action, namely agonism, antagonism and potentiation, occur at the level of the cell (*in vitro;* chapter 2, 3*)*, isolated tissue (*ex vivo;* chapter 4) and complete animal (*in vivo;* chapter 5). In addition some specific differences in responses between the assays were revealed, which may help explaining how these compounds elicit their effects on T_3-action. The signal transduction pathway of T_3

is a complex interplay, as described in chapter 1 in more detail, and in this paragraph possible mechanisms for disruption of this pathway are discussed based on the indications obtained from the various assays described in this thesis.

Agonistic compounds

Only compounds with a T_3-like structure such as the T_3-like BDE-OH and to a lesser degree the T_4-like BDE-OH, were able to induce effects in the T-screen in absence of T_3 (chapter 2). Also, none of the 27 brominated flame retardants tested within the framework of the EU-FIRE project induced a response in the T-screen to a significant degree. This suggests that the structural requirements for a fit into the tight TR-pocket are strictly T_3-like as has been suggested before (Dietrich et al., 1977). Indeed, to date the pharmaceutical industry has had limited success with the design of specific TRα and TRβ agonists (Chiellini et al., 1998; Ye et al., 2003; Yoshihara et al., 2003). T_3-like compounds may be formed upon metabolism of halogenated compounds with a biphenyl or diphenylether backbone and this mechanism is an interesting topic for further research on the biosynthesis of T_3 agonists.

Antagonistic compounds

Although compounds have to be T_3-like to a high degree to be able to bind the TR and activate post-receptor gene cascades, a non T_3-like compound such as BDE206 antagonized T_3-action (chapter 2-5). This might imply that such compounds occupy the TR or the Thyroid Responsive Element (TRE) and subsequently inhibit T_3-action, which ultimately leads to gene suppression. It has previously been shown that 4'-hydroxy-2,3,3',4,5-pentachlorobiphenyl (PCB106-OH) disrupts TRβ binding to a TRE because the TR-RXR heterodimer complex is partially dissociated from the TRE in the presence of PCB106-OH (Miyazaki et al., 2004), possibly via steric hindrance. An alternative mechanism underlying the antagonistic effects of BDE206 can be sought in binding to an allosteric site on the TR, which is also a site targeted by other endogenous factors such as cofactors

(McKenna and O'Malley, 2002). In a mammalian two-hybrid assay bisphenol-A (BPA) was found to recruit the nuclear co-repressor N-CoR to the TR, thus suppressing its transcriptional activity (Moriyama et al., 2002). Finally, the role of cellular TH membrane transporters needs to be considered (Friesema et al., 2005). Possibly compounds such as BDE206 inhibit cellular T_3-uptake, and such a mechanism remains an interesting topic for further research.

Potentiating compounds

The potentiating effects of compounds observed in this thesis can be divided in two groups: DIB and BDE28 with a biphenyl/diphenylether backbone being active in the *in vitro* TRα/TRβ specific assay in CV-1 cells without endogenous TRs (chapter 3), and HBCD with a very different molecular backbone (chapter 3; Figure 1) that only potentiated T_3-action in *in vitro/ex vivo* models with endogenous TRs (chapter 2, 4 and 5). An important difference between the latter *in vitro/ex vivo* models and the *in vitro* TRα/β specific reporter gene assays, is that the CV-1 cells used in this assay do not contain endogenous TRs, and therefore possibly also lack related specific sites that can be targeted by HBCD. This suggests that potentiation of T_3-action observed earlier for HBCD, occurs via a different mechanism than for DIB and BDE28 which more closely resemble the molecular structure of T_3. For example, HBCD may affect cell proliferation of GH_3 cells in the T-screen by influencing cell-specific cell cycle regulators such as cyclin or cyclin-dependent kinase levels and their activity (Barrera-Hernandez et al., 1999). Potentiation of compounds in general could be induced by enhanced recruitment of receptor-coactivators which are important for the activation of the TR-RXR complex. It has been shown before that overexpression of the cofactor steroid receptor coactivator-1 (SRC-1) in MCF-7 breast cancer cells results in a increase in the mitogenic response to estrogen (Tai et al., 2000). Thus, the sensitivity of a cell to a specific level of a hormone may be enhanced by the availability of SRC-1. Enhanced SRC-1 recruitment by compounds, either *in vitro* or *in vivo*, however, has not yet been reported and deserves more investigation.

Another hypothesis is that potentiating compounds enhance the availability of T_3, either by inducing TH membrane transporters or by inducing deiodinase activity, thus activating the transformation of T_4 to T_3.

7.4 Perspectives for the use of the mechanism based *in vitro* and *in vivo* assays in environmental toxicological research

The assays presented in this thesis are very promising complementary *in vitro* and *in vivo* tools to quantify the effects of compounds on T_3-action. The advantages, disadvantages and perspectives of those assays are discussed below.

T-screen

The T-screen is a very rapid and simple functional *in vitro* assay to determine agonistic, antagonistic and potentiating effects of compounds on T_3-action (chapter 2). It is important to test compounds in the absence and presence of T_3 while combination effects frequently occurred. In addition to TR-mediated effects, the T-screen incorporates effects on other cellular pathways that are not yet elucidated but also occur *in vivo*. Until now no false negatives or positives have been found for parent compounds interacting via the TR-mediated T_3-action. The rat pituitary (GH_3) cell line, however, lacks CYP-activity, which may result in false-negative responses for compounds that could be "bioactivated" to active compounds in the *in vivo* situation. In that case an additional biotransformation step is necessary. In addition, a false positive result could be expected for compounds interfering with cell proliferation via other than TH-mediated mechanisms. As the T-screen is a functional assay, the EC_{50} values obtained with the T-screen are more relevant for the *in vivo* situation than EC_{50} data obtained with TR binding studies. The assay also has been applied as a tool to detect thyroid hormone disrupting compounds in sediment extracts (Gutleb et al., 2005). Since GH_3 cells express both TR isoforms (Hahn et al., 1999; Ball et al., 1997), it is not possible to predict differential tissue specific effects.

TRα/β specific reporter gene assays

The TRα/β specific reporter gene assays offer the possibility to study the TR-isoform specific effects of compounds. Although in its present form it is less rapid and easy to perform than the T-screen, luciferase production is a specific reflection of the affinity of a compound to interfere with TR-mediated transactivation (chapter 3). Even in the small panel of tested compounds differences were found in effects via the TRα or TRβ . This type of information greatly increases insight in the mechanisms underlying tissue-specific effects. In vertebrates, including humans, TR isoforms are differentially expressed in tissues such as the brain and skeletal muscle (Lazar, 1993), and TRα or TRβ-specific information allows determination of a potential risk for specific endpoints. Comparable receptor isoform selectivity has been demonstrated for endocrine disrupting compounds differentially activating the estrogen receptor α and β (ter Veld et al., 2006). As the transient transfection of cells is a laborious and expensive procedure, the development of a stabile cell line expressing either TRα or TRβ is required to make the assay suitable for high throughput analysis. Furthermore, the incorporation of specific CYP-expression vectors will provide useful information about metabolization of compounds to more potent metabolites.

Ex vivo tail tip assay

The *ex vivo* tail tip assay offers great possibilities in studying the interactive effects of compounds on T_3-action in primary tissues that have not been genetically modified or immortalized such as most cancer cell lines (chapter 4). Furthermore, influences such as hormonal feed-back mechanisms, biotransformation and effects via the thyroid gland or other hormones such as corticoids (Hayes, 1997) can be excluded. Since tadpoles are not euthanized as done in previous studies (Tata, 1966; Iwamuro et al., 2003) and regenerate their tail tips, this assay is an improvement in terms of reduction of animal use. More research should be conducted to simplify the method of quantifying compound-induced effects. As of yet, tail tip regression is quantified by measuring tail tip length on a millimetre-

grid. This procedure, however, is very laborious and susceptible to inter-observer variance, the use of enzymatic endpoints, such as caspase-3 activity (Gurtu et al., 1997) or collagenase-3 up/down regulation as a molecular endpoint (Lim et al., 2002) may be very useful.

In vivo proliferation assay

A limitation of all *in vitro* (cellular) and *ex vivo* (organ/tissue culture) systems is that only effects occurring in the absence of other endogenous hormones, growth factors, other cell-cell interactions, etc. can be detected. Therefore the effects after *in vivo* exposure may differ greatly from the results obtained in tissue or cell culture. The *in vivo* proliferation assay using one-week old *Xenopus laevis* tadpoles as performed in this thesis is almost as sensitive to T_3 as the T-screen and allows studying tissue-specific (brain and rostral head region) effects of T_3 and TH-disrupting compounds in a full organism (chapter 5). As one-week old tadpoles lack circulating T_3 but are highly sensitive to the T_3-stimulation, it is important to test compounds in combination with T_3 as well in order to predict the effects at later developmental stages. As T_3 levels rise towards the onset of metamorphosis (Leloup and Buscaglia, 1977) it would be interesting to test effects of TH disrupting compounds via this technique in later developmental stages. Unfortunately the *in vivo* proliferation assay with *Xenopus laevis* tadpoles cannot be performed in later developmental stages due to the reduced permeability of the tadpoles for the anti-phosphohistone H3 antibody and the increased pigmentation of the tadpoles making it impossible to quantify the fluorescence in the intact organism at later developmental stages. A solution could be to use tissue sections of older treated animals or perhaps use unpigmented albino *Xenopus tropicalis*. Also the development of a TR-specific transgenic *in vivo* Xenopus model, comparable to e.g. the ER-specific transgenic zebrafish for detecting estrogens (Legler et al., 2000), would be an interesting *in vivo* model for further research.

When applying the *in vivo* proliferation assay it became clear how important it is to optimize the exposure- and concentration-period, as higher or longer exposure reduces proliferation, probably due to cell differentiation or apoptosis. Easy accessible tissues, such as the rostral head region, appeared to reach their maximum proliferation sooner than less accessible tissue such as brain. Also transport of the test compounds to the tissue may differ depending on physico-chemical properties. As of yet, only a small panel of compounds have been tested in two tissues of the young tadpoles. Although more research should be conducted on optimization and validation of this assay, the effects of the tested compounds on T_3-induced cell proliferation in those tissues showed a high correlation with the *in vitro* proliferation assay suggesting that the effects in such young life-stages may be predictable based on the T-screen results.

Multispecies Freshwater Biomonitor (MFB)

The Multispecies Freshwater Biomonitor (MFB) was successfully applied in this thesis (chapter 6) to quantify behavioral responses of amphibian tadpoles exposed to toxic compounds. Behavioral responses usually are very sensitive as they are an integration of effects at several physiological endpoints (Scherer, 1992), and even changes in subtle tadpole movements, such as tadpole tail tip oscillations, could automatically be quantified. Although behavioural responses are still effects at the individual level, it has been shown that these effects can be extrapolated to consequences at a higher level of biological organization, for example reduced predator avoidance (Raimondo et al., 1998; Zhou et al., 1998). In the present thesis the effects of a model compound (triphenyltin) on tadpole behavior was quantified with the MFB. As some thyroid hormone disrupting compounds have been shown to affect tadpole metamorphosis (Gutleb et al., 1999), and tadpole metamorphosis is accompanied by behavioural changes (chapter 6), it would be very interesting to further study the effects of thyroid hormone disrupting compounds on tadpole behaviour during the course of metamorphosis and further validate the sensitivity of this *in vivo* assay.

7.5 Possible significance of the thyroid hormone disrupting effects revealed in this thesis

The research presented in this thesis clearly shows that environmental contaminants can interfere with TR-mediated T_3-action. A question that remains to be answered is how man and wildlife could be affected by these compounds. In this paragraph both the extrapolation between vertebrate species as well as the consequences of current levels of known TH-disrupting compounds in biota and the environment are briefly discussed.

Extrapolation between vertebrate species

As is depicted in Figure 2, the temporal profiles of plasma TH levels in amphibians and humans during embryonic, fetal and postnatal/post-metamorphic developmental stages are very similar (Leloup and Buscaglia, 1977; Tata, 1999; Shi et al., 2002).

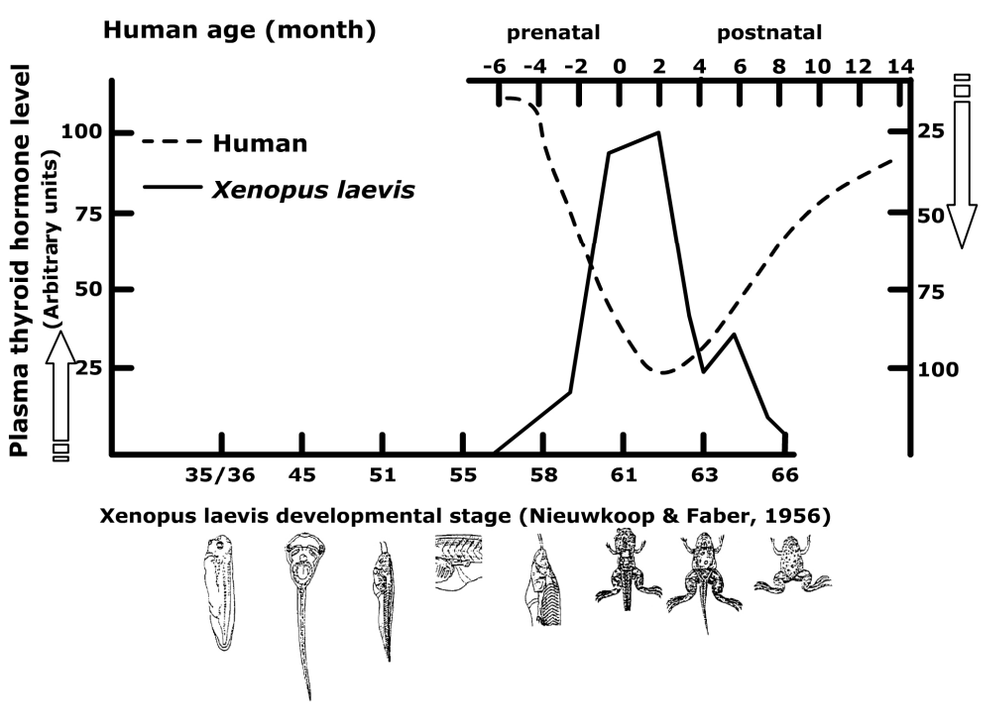


Figure 2. Natural course of T_3 plasma levels during human and *Xenopus laevis* development. The human T_3-curve is based on Tata (1993) and scaled based on developmental stages compared to those of *Xenopus laevis* which are based on data of Leloup and Buscaglia (1977). Figure reproduced with modifications from Shi et al., 2002.

During the first trimester, in which human tissue formation takes place, the T_3–levels in the serum are very low, but T_3 levels rise (from 0.125 nM to 1 nM) during maturation of the thyroid gland in the second trimester of development (Porterfield and Hendrich, 1993; Koopman-Esseboom et al., 1994). This implicates that the human fetus in this particular stage of development may be very sensitive to compounds that potentiate or antagonize T_3-action, just as pre- and pro-metamorphic tadpoles (Leloup and Buscaglia, 1977). As thyroid hormone receptors are highly conserved between various vertebrate species (Yaoita et al., 1990), the assays presented in this thesis are expected to be very relevant for studying possible adverse effects on not only amphibian development, but also on human and other vertebrate early development. The various vertebrate species may even share a comparable tissue distribution of the different TR isoforms, although specific differences will exist, for example because of the occurrence of tail resorption in metamorphosing amphibians.

Possible consequences of exposure to current levels of known TH-disrupting compounds

T_3-like (and T_4-like) compounds as agonists

Animals and humans are exposed to mixtures of TH-disrupting compounds such as PBDEs, PCBs and other persistent organic pollutants (POPs) (Ross and Birnbaum, 2003). In addition, depending on the species and type of compound, they may be metabolized into TH-disrupting hydroxy metabolites. Such compounds have been shown to mimic T_4 and bind to TTR in mammalian blood (Meerts et al., 2000), and as a consequence some of these compounds have been found to accumulate selectively in the blood. It was even shown in human plasma that the concentration of 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl (PCB107-OH) was 0.36 $\mu g.g^{-1}$ lipid (5.2 nM) which was comparable to that of the parent PCB congeners (Bergman et al., 1994).

The results presented in this thesis show that hydroxylated BDEs, which resemble the structure of hydroxylated PCBs, can mimic or potentiate the action of T_3 in the

nanomolar range (chapter 2). Especially the T_3-like BDE-OH was a potent T_3-agonist with a no observed effect concentration (NOEC) of 50 nM, which is only 10 times higher than the concentration found in human blood for single PCB-OH-metabolites as reported by Bergman and co-workers (1994). As not single compounds, but mixtures of OH-metabolites and parent compounds exist, and TH-disrupting compounds have been shown to affect various mechanisms (chapter 1.4), the margin of safety (MOS) for TH-disruption could be very small or absent. Such data suggest that PCBs, PBDEs, other POPs and their metabolites may be able to exert adverse effects on early thyroid hormone dependent developmental processes such as vertebrate brain development and tissue differentiation.

BDE206 as antagonist

The nonaBDE (BDE206), which was a potent antagonist of T_3-action in the studies presented in this thesis (chapters 2, 3, 4 and 5), has only been recently been synthesized (Christiansson et al., 2006) and detected in biological matrices such as human serum (Thuresson et al., 2006). In addition, there are several lines of evidence that demonstrate the debromination of decaBDE (BDE209) by abiotic environmental factors (Soderstrom et al., 2004), micro-organisms (Gerecke et al., 2005) or aquatic biota (Kierkegaard et al., 1999; La Guardia et al., 2004; Stapleton et al., 2004) to lower brominated BDEs, including BDE206. Furthermore, behavioral effects were observed with 3-day old pups of BDE209 exposed mice (Viberg et al., 2003) which were ascribed to BDE206 and other possible metabolites of BDE209. As the environmental levels of BDE209 are rising (Zegers et al., 2003), and this compound has been detected in breast milk from nursing mothers in Texas (USA) up to a concentration of 8.24 $ng.g^{-1}$ lipid (146 pM) (Schecter et al., 2003), more studies on the toxicity of major BDE209 metabolites should be initiated. Also, further research is necessary to demonstrate maternal transfer to the fetus of BDE209 and/or its metabolites. It has been shown that BDEs, like PCBs and some other organochlorine compounds, are able to cross the

placenta into the fetal circulation (Hites et al., 2003). Because of the lipophilic characteristics of BDEs, these compounds are deposited in maternal adipose tissue and when body fat is used, redistribution, biotransformation to hydroxylated metabolites and transfer to the fetal compartment may take place.

HBCD, BDE28 and DIB as potentiating compounds

As of yet, it is too early to predict the possible consequences of the observed potentiating effects of HBCD, BDE28 and DIB. Of these compounds HBCD is the most relevant, considering high production volumes (2001: 16700 tonnes) (Law et al., 2005) and increasing concentrations in biota and the environment (Heeb et al., 2005). HBCD significantly potentiated T_3-action at a concentration of 100 nM in the T-screen (chapter 2), *ex vivo* tail tip assay (chapter 4) and *in vivo* proliferation assay (chapter 5). This experimental level of HBCD is much higher than levels detected in cord blood (representing fetal blood) (<0.6 – 14 pM), Dutch mothers serum (<2 – 84 pM) (Weis et al., 2004) or breast milk from Swedish mothers (14 - 150 pM) (Lopez et al., 2004). It is very relevant to further elucidate the mechanistic action and impact of HBCD, which could have implications for risk assessment of this compound. Since the fetus is especially sensitive to small changes in TH-levels, it is also relevant to investigate if HBCD accumulates in the fetus. Although probably none of the compounds tested for their TR-mediated disruption of T_3-action are present in the environment at effect levels, they are part of a large mixture of thyroid hormone disrupting compounds (Weis et al., 2004; Lopez et al., 2004) including their OH-metabolites. Furthermore, the effects of thyroid hormone disrupting compounds have been shown to be additive (Crofton et al., 2005), and several sites that control TH-homeostasis may be affected at the same time. Exposure of the vulnerable developing fetus to complex mixtures of compounds may result in significant effects on vertebrate development. As also TH excess can impair embryogenesis which can be positively correlated with an increased rate of miscarriages and/or a lower birth weight of unaffected neonates (Anselmo et al., 2005), also potentiating effects

could have serious consequences. Therefore it is important to assess the effect of realistic mixtures of TH-disrupting compounds including their metabolites, and study further how effects of such compounds should be taken into account.

7.6 Main conclusions

This thesis was focusing on three research objectives. Therefore the main conclusions based on this research are presented in relation to these three objectives.

1. Development and validation of *in vitro* and *in vivo* assays for disruption of thyroid hormone action.
2. Elucidation of the potential mechanisms of TR-mediated disruption.
3. Advice whether predictions can be made based on *in vitro* studies for *in vivo* disruption of thyroid hormone action.

Objective 1:

- The T-screen is a functional assay for identification of compounds with agonistic, antagonistic and potentiating TR-mediated effects. The T-screen did not show false positive or false negative responses, but cannot distinguish TR isoform-specific effects of the compounds tested. Therefore the T-screen is a very useful first step in the screening of compounds for their TR-mediated TH-disruption. This first screening should be performed in presence and absence of T_3 at its EC_{50} to be able to detect agonists, antagonists and potentiating compounds.

- The transient TR-isoform selective reporter gene assay demonstrates that environmental contaminants can interfere with T_3-induced activation of TRα and/or TRβ. This is relevant to predict or explain tissue- or life stage specific effects of such compounds for the *in vivo* situation. The potentiating effect of HBCD could not be picked up by this TR-specific assay. This assay (preferably as stable reporter gene assay) is most suitable to further study the compounds that were active in the T-screen.

- The *in vivo* proliferation assay and *ex vivo* tail tip assay were suitable to validate the *in vitro* assay responses and to study specific TR-mediated effects in more detail. They are too time-consuming to be used in high throughput hazard assessment of TR-mediated TH-disrupting compounds.

- The Multispecies Freshwater Biomonitor (MFB) is suitable to automatically quantify even subtle changes in tadpole behavior and physiology. As tadpole behavior has been shown to change during the course of metamorphosis, it remains interesting to study the integrated effects of sublethal effects of TH-disrupting compounds on amphibian behavior during metamorphosis.

Objective 2:

- Application of the combination of assays as was done in this thesis clearly revealed how compounds could be agonists or antagonists via the TRα and/or TRβ. Only compounds that are strictly T_3-like, such as the T_3-like BDE-OH, are able to induce effects in the assays in absence of T_3. Such compounds may be formed upon biotransformation of parent compounds. Non T_3-like compounds, such as BDE206, may antagonize T_3-action by occupation of the TR or TRE, but other mechanisms such as interference/interaction with cofactors which may ultimately lead to gene suppression, cannot be excluded. Potentiation of T_3-action was found to occur in a TR-related manner for BDE28 and DIB. The potentiation of T_3-action by HBCD does not seem to occur via direct TR-activation, and the mechanism still remains to be further elucidated.

Objective 3:

- The bioassays presented in this thesis are very promising complementary *in vitro*, *ex vivo* and *in vivo* biomarkers to quantify the effects of compounds on T_3-action. Based on the results obtained, the *in vitro* results are highly

predictive for the effects as observed *in vivo*. The fact that compounds can be tested in the presence of T_3 is important, since in the *in vivo* situation also physiological concentrations of T_3 can be present.

References

Anselmo, J., Cao, D., Karrison, T., Weiss, R.E., Refetoff, S., 2004. Fetal loss associated with excess thyroid hormone exposure. **Jama 292**, 691-695.

Barrera-Hernandez, G., Park, K.S., Dace, A., Zhan, Q., Cheng, S.Y., 1999. Thyroid hormone-induced cell proliferation in GC cells is mediated by changes in G1 cyclin/cyclin-dependent kinase levels and activity. **Endocrinology 140**, 5267-5274.

Bergman, A., Klasson-Wehler, E., Kuroki, H., 1994. Selective retention of hydroxylated PCB metabolites in blood. **Environ Health Persp 102**, 464-469.

Brouwer, A., Morse, D.C., Lans, M.C., Schuur, A.G., Murk, A.J., Klasson-Wehler, E., Bergman, A., Visser, T.J., 1998. Interactions of persistent environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. **Toxicol Ind Health 14**, 59-84.

Champlin, D.T., Truman, J.W., 1998. Ecdysteroid control of cell proliferation during optic lobe neurogenesis in the moth *Manduca sexta*. **Development 125**, 269-277.

Chiellini, G., Apriletti, J.W., Yoshihara, H.A., Baxter, J.D., Ribeiro, R.C., Scanlan, T.S., 1998. A high-affinity subtype-selective agonist ligand for the thyroid hormone receptor. **Chem Biol 5**, 299-306.

Christiansson, A., Teclechiel, D., Eriksson, E., Bergman, A., Marsh, G., 2006. Methods for the synthesis of nonabromodiphenyl ethers and a chloro-nonabromodiphenyl ether. **Chemosphere 63**, 562-569.

Dietrich, S.W., Bolger, M.B., Kollman, P.A., Jorgensen, E.C., 1977. Thyroxine analogues. 23. Quantitative structure-activity correlation studies of *in vivo* and *in vitro* thyromimetic activities. **J Med Chem 20**, 863-880.

Friesema, E.C.H., Jansen, J., Milici, C., Visser, T.J. 2005. Thyroid Hormone Transporters. Vitamins & Hormones: Academic Press. p 137.

Furlow, J.D., Yang, H.Y., Hsu, M., Lim, W., Ermio, D.J., Chiellini, G., Scanlan, T.S., 2004. Induction of larval tissue resorption in *Xenopus laevis* tadpoles by the thyroid hormone receptor agonist GC-1. **J Biol Chem 279**, 26555-26562.

Gerecke, A.C., Hartmann, P.C., Heeb, N.V., Kohler, H.P.E., Giger, W., Schmid, P., Zennegg, M., Kohler, M., 2005. Anaerobic degradation of decabromodiphenyl ether. **Environ Sci Technol 39**, 1078-1083.

Gurtu, V., Kain, S.R., Zhang, G., 1997. Fluorometric and colorimetric detection of caspase activity associated with apoptosis. **Anal Biochem 251**, 98-102.

Gutleb, A.C., Appelman, J., Bronkhorst, M.C., van den Berg, J.H.J., Spenkelink, A., Brouwer, A., Murk, A.J., 1999. Delayed effects of pre- and early-life time exposure to polychlorinated biphenyls on tadpoles of two amphibian species (*Xenopus laevis* and *Rana temporaria*). **Environ Toxicol Phar 8**, 1-14.

Gutleb, A.C., Meerts, I.A.T.M., Bergsma, J.H., Schriks, M., Murk, A.J., 2005. T-Screen as a tool to identify thyroid hormone receptor active compounds. **Environ Toxicol Phar 19**, 231-238.

Hamers, T., Kamstra, J.H., Sonneveld, E., Murk, A.J., Kester, M.H.A., Andersson, P. L., Legler, J., Brouwer, A., 2006. *In vitro* profiling of the endocrine disrupting potency of brominated flame retardants. **Toxicol Sci 92**, 157-173.

Hayes, T.B., 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. **Am Zool 37**, 185-194.

Heeb, N.V., Schweizer, W.B., Kohler, M., Gerecke, A.C., 2005. Structure elucidation of hexabromocyclododecanes - a class of compounds with a complex stereochemistry. **Chemosphere 61**, 65-73.

Hites, R.A., Dodder, N.G., Mazdai, A., Abernathy, M.P., Bigsby, R.M., 2003. Polybrominated diphenylethers in maternal and fetal blood samples. **Organohalogen Compounds 61**, 5-8.

Iwamuro, S., Sakakibara, M., Terao, M., Ozawa, A., Kurobe, C., Shigeura, T., Kato, M., Kikuyama, S., 2003. Teratogenic and anti-metamorphic effects of bisphenol A on embryonic and larval *Xenopus laevis*. **Gen Comp Endocr 133**, 189-198.

Kierkegaard, A., Balk, L., Tjarnlund, U., De Wit, C. A., Jansson, B., 1999. Dietary uptake and biological effects of decabromodiphenyl ether in rainbow trout (*Oncorhynchus mykiss*). **Environ Sci Technol 33**, 1612-1617.

Koopman-Esseboom, C., Huisman, M., Weisglas-Kuperus, N., Boersma, E.R., de Ridder, M.A., Van der Paauw, C.G., Tuinstra, L.G., Sauer, P.J., 1994. Dioxin and PCB levels in blood and human milk in relation to living areas in The Netherlands. **Chemosphere 29**, 2327-2338.

La Guardia, M.J., Hale, R.C., Harvey, E., 2004. Environmental debromination of decabrominated diphenyl ether. Proc. The Third International Workshop on Brominated Flame Retardants (BFR2004), University of Toronto, Toronto, Ontario, Canada.

Law, R.J., Kohler, M., Heeb, N.V., Gerecke, A.C., Schmid, P., Voorspoels, S., Covaci, A., Becher, G., Janak, K., Thomsen, C., 2005. Hexabromocyclododecane challenges scientists and regulators. **Environ Sci Technol 39**, 281A-287A.

Lazar, M.A., 1993. Thyroid hormone receptors: multiple forms, multiple possibilities. **Endocr Rev 14**, 184-193.

Legler, J., Broekhof, J.L.M., Brouwer, A., Lanser, P.H., Murk, A.J., Van der Saag, P. T., Vethaak, A.D., Wester, P., Zivkovic, D., Van der Burg, B., 2000. A novel *in vivo* bioassay for (xeno-) estrogens using transgenic zebrafish. **Environ Sci Technol 34**, 4439-4444.

Leloup, J., Buscaglia, M., 1977. Triiodothyronine, Hormone Of Amphibian Metamorphosis. **Comptes Rendus Hebdomadaires Des Seances De L'Academie Des Sciences Serie D 284**, 2261-2263.

Lim, W., Nguyen, N.-H., Yang, H.Y., Scanlan, T.S., Furlow, J.D., 2002. A Thyroid Hormone Antagonist That Inhibits Thyroid Hormone Action in Vivo. **J Biol Chem 277**, 35664-35670.

Lopez, D., Athanasiadou, M., Athanassiadis, I., Yáñez Estrada, L., Díaz-Barriga, F., Bergman, A., 2004. A Preliminary Study on PBDEs and HBCDD in Blood and Milk from Mexican Women. The Third International Workshop on Brominated Flame Retardants, BFR 2004.

McKenna, N.J., and O'Malley, B.W., 2002. Minireview: Nuclear receptor co-activators - an update. **Endocrinology 143**, 2461-2465.

Meerts, I.A., van Zanden, J.J., Luijks, E.A., van Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A., Brouwer, A., 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. **Toxicol Sci 56**, 95-104.

Miyazaki, W., Iwasaki, T., Takeshita, A., Kuroda, Y., Koibuchi, N., 2004. Polychlorinated biphenyls suppress thyroid hormone receptor-mediated transcription through a novel mechanism. **J Biol Chem 279**, 18195-18202.

Moriyama, K., Tagami, T., Akamizu, T., Usui, T., Saijo, M., Kanamoto, N., Hataya, Y., Shimatsu, A., Kuzuya, H., Nakao, K., 2002. Thyroid hormone action is disrupted by bisphenol A as an antagonist. **J Clin Endocrinol Metab 87**, 5185-5190.

Porterfield, S.P., Hendrich, C.E., 1993. The role of thyroid hormones in prenatal and neonatal neurological development-current perspectives. **Endocr Rev 14**, 94-106.

Raimondo, S.M., Rowe, C.L., 1998. Exposure to coal ash impacts swimming performance and predator avoidance in larval bullfrogs (*Rana catesbeiana*). **J Herpetol 32**, 289-292.

Ross, P.S., Birnbaum, L.S., 2003. Integrated human and ecological risk assessment: A case study of persistent organic pollutants (POPs) in humans and wildlife. **Hum Ecol Risk Assess 9**, 303-324.

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

Scherer, E., 1992. Behavioral-Responses as Indicators of Environmental Alterations - Approaches, Results, Developments. **Journal of Appl Ichthyol 8**, 122-131.

Schreiber, A.M., Das, B., Huang, H., Marsh-Armstrong, N., Brown, D.D., 2001. Diverse developmental programs of *Xenopus laevis* metamorphosis are inhibited by a dominant negative thyroid hormone receptor. **P Natl Acad Sci USA 98**, 10739-10744.

Schriks, M., Vrabie, C.M., Gutleb, A.C., Faassen, E.J., Rietjens, I.M.C.M., Murk, A.J., 2006. T-screen to quantify functional potentiating, antagonistic and thyroid hormone-like activities of poly halogenated aromatic hydrocarbons (PHAHs). **Toxicol In Vitro 20**, 490-498.

Shi, Y.-B., Ritchie, J.W.A., Taylor, P.M., 2002. Complex regulation of thyroid hormone action: multiple opportunities for pharmacological intervention. **Pharmacol Therapeut 94**, 235-251.

Soderstrom, G., Sellstrom, U., de Wit, C.A., Tysklind, M., 2004. Photolytic debromination of decabromodiphenyl ether (BDE 209). **Environ Sci Technol 38**, 127-132.

Stapleton, H.M., Alaee, M., Letcher, R.J., Baker, J.E., 2004. Debromination of the flame retardant decabromodiphenyl ether by juvenile carp (*Cyprinus carpio*) following dietary exposure. **Environ Sci Technol 38**, 112-119.

Tai, H., Kubota, N., Kato, S., 2000. Involvement of nuclear receptor coactivator SRC-1 in estrogen-dependent cell growth of MCF-7 cells. **Biochem Biophys Res Commun 267**, 311-316.

Tata, J.R., 1966. Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. **Dev Biol 13**, 77-94.

Tata, J.R., 1999. Amphibian metamorphosis as a model for studying the developmental actions of thyroid hormone. **Biochimie 81**, 359-366.

Thuresson, K., Bergman, A., Rothenbacher, K., Herrmann, T., Sjolin, S., Hagmar, L., Papke, O., Jakobsson, C., 2006. Polybrominated diphenyl ether exposure to electronics recycling workers – a follow up study. **Chemosphere 64**, 1855-1861.

Veld, ter M.G.R., Schouten, B., Louisse, J., Es, van D.S., Saag, van der P.T., Rietjens, I.M.C.M., Murk, A.J., 2006. Estrogenic potency of food packaging associated plasticizers and antioxidants as detected in ERα and ERβ-reporter cell lines. **J Agr Food Chem 54**, 4407-4416.

Viberg, H., Fredriksson, A., Jakobsson, E., Orn, U., Eriksson, P., 2003. Neurobehavioral derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development. **Toxicol Sci 76**, 112-120.

Weber, R. 1967. Biochemistry of amphibian metamorphosis. Weber R, editor. New York: Academic press.

Weis, J.S., Meijer, L., Sauer, P., Linderholm, L., Athanassiadis, I., Bergman, A., 2004. PBDE and HBCDD levels in blood from Dutch mothers and infants - Analysis of a Dutch Groningen infant cohort. **Organohalogen Compounds 66**.

Yaoita, Y., Shi, Y., Brown, D., 1990. *Xenopus laevis* {alpha} and {beta} Thyroid Hormone Receptors. **P Natl Acad Sci USA 87**, 7090-7094.

Ye, L., Li, Y. L., Mellstrom, K., Mellin, C., Bladh, L. G., Koehler, K., Garg, N., Garcia Collazo, A. M., Litten, C., Husman, B. et al., 2003. Thyroid receptor ligands. 1. Agonist ligands selective for the thyroid receptor beta1. **J Med Chem 46**, 1580-1588.

Yoshihara, H.A.I., Apriletti, J.W., Baxter, J.D., Scanlan, T.S., 2003. Structural determinants of selective thyromimetics. **J Med Chem 46**, 3152-3161.

Zegers, B.N., Lewis, W.E., Booij, K., Smittenberg, R.H., Boer, W., de Boer, J., Boon, J.P., 2003. Levels of polybrominated diphenyl ether flame retardants in sediment cores from Western Europe. **Environ Sci Technol 37**, 3803-3807.

Zhou, T., Weis, J.S., 1998. Swimming behaviour and predator avoidance in three populations of *Fundulus heteroclitus* larvae after embryonic and/or larval exposure to methylmercury. **Aquat Toxicol 43**, 131-148.

Zoeller, R.T., 2005. Environmental chemicals as thyroid hormone analogues: New studies indicate that thyroid hormone receptors are targets of industrial chemicals? **Mol Cell Endocrinol 242**, 10-15.

Samenvatting

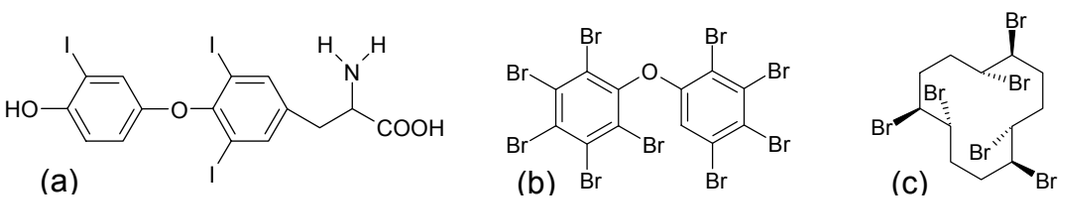
De laatste jaren is er toenemende bezorgdheid over de mogelijke effecten van milieuverontreinigende stoffen op het functioneren van hormonen in mens en dier. Op dit moment richt het onderzoek en beleid naar zulke hormoonverstorende stoffen zich echter vooral op verstoring door stoffen van de geslachtshormoon huishouding. Veel minder aandacht gaat uit naar verstoring van het schildklier- oftewel thyroïdhormoon (TH) systeem. Toch blijkt uit verschillende onderzoeken dat dit belangrijke hormoonsysteem kwetsbaar is voor verstoring door hormoonverstorende stoffen (Brouwer et al., 1998). Verstoring van de TH huishouding kan leiden tot een scala van ongewenste effecten op ontwikkeling, groei en de energiehuishouding van gewervelde dieren zoals zoogdieren en amfibiëen (hoofdstuk 1.4). Recent onderzoek wijst nu uit dat de thyroïdhormoon receptoren (TR's) in de cel doelwit kunnen zijn van hormoonverstorende stoffen (Zoeller et al., 2005). Helaas zijn effecten die via de TR verlopen moeilijk aan te tonen omdat geschikte testmethoden schaars zijn. Er is meer aandacht voor onderzoek naar andere belangrijke aangrijpingspunten zoals het transport van TH in het bloed, afbraak en activatie van TH.

Doel van dit onderzoek

Het in dit proefschrift beschreven onderzoek heeft op de eerste plaats als doel de ontwikkeling van nieuwe testsystemen voor de bepaling TH verstoring door chemische stoffen op het niveau van de TR. Deze testsystemen maken gebruik van cellen die gekweekt worden in kweekmedium (*in vitro*), levende dieren (*in vivo*) of van stukjes geïsoleerd staartweefsel afkomstig van kikkervisjes (*ex vivo*). Op de tweede plaats is het doel het vergroten van inzicht in de mechanismen waarlangs hormoonverstorende stoffen de TR gemedieerde werking van TH kunnen verstoren. Ten derde wordt bepaald in hoeverre de effecten van de hormoonverstorende stoffen, gemeten met de de *in vitro* testsystemen, de effecten kunnen voorspellen voor de *in vivo* situatie.

Onderzochte stoffen

Naast een aantal controle-stoffen die bekend staan om hun TR-stimulerende (diidodo biphenyl, DIB) en hun TR-remmende werking (amiodarone) is ook een aantal moeilijk afbreekbare milieuverontreinigende stoffen gebruikt. Het grootste gedeelte van deze milieuverontreinigende stoffen behoren tot de groep van de gebromeerde vlamvertragers. Deze stoffen worden aan producten zoals textiel en automaterialen toegevoegd, om ze beter te beschermen tegen brand (de Wit, 2002). De meest toegepaste vlamvertragers zijn oa. de gebromeerde diphenylethers (BDEs) en hexabromocyclododecane (HBCD) (Figuur 1). Daarnaast zijn drie verschillende BDEs getest die sterke gelijkenis hebben met de actieve vorm van TH (T_3), de transportvorm (T_4) en een afbraakproduct (T_2) (Figuur 2b).

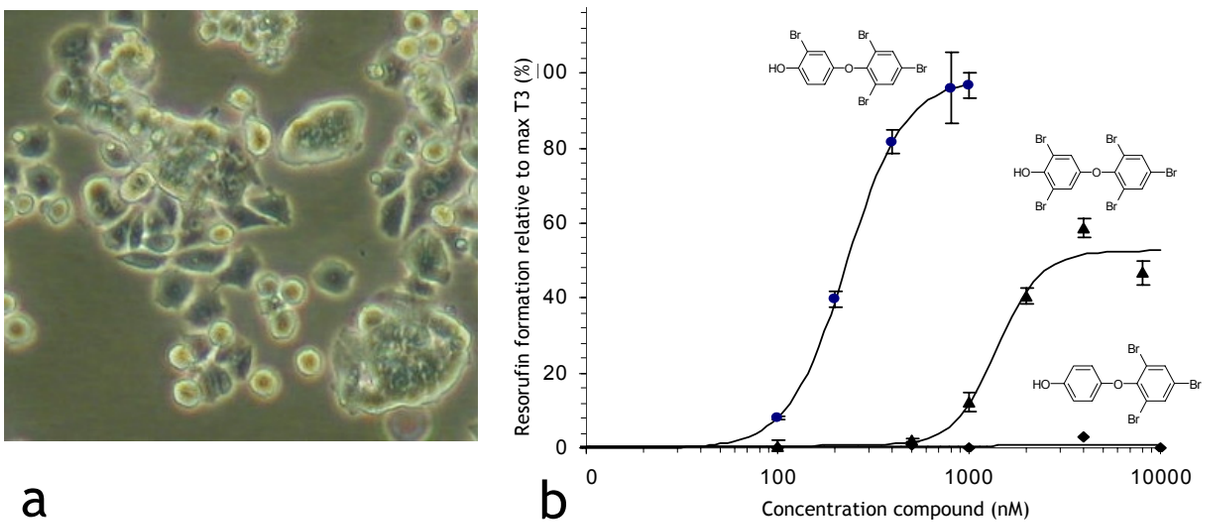


Figuur 1. Chemische structuren van (a) 3,3',5-triiodo-L-thyronine (T_3) (b) 2,2'3,3',4,4',5,5',6-nonaBDE (BDE206) en (c) hexabromocyclododecane (HBCD).

Overzicht van de toegepaste testsystemen en de belangrijkste conclusies

1. T-screen (hoofdstuk 2)

De T-screen wordt uitgevoerd met een ratten hypofyse cellijn (GH_3) (Figuur 2a), die uitsluitend groeit in aanwezigheid van de actieve vorm van TH (T_3). De groeistimulerende werking van T_3 loopt via activatie van in de cel aanwezige TR's. De mate waarin stoffen de celgroei kunnen stimuleren, remmen of versterken wordt bestudeerd in aan- en afwezigheid van T_3.



Figuur 2. a) Microscopische foto van de ratten hypofyse cellijn(GH_3). b) Effecten van de T_2-achtige (♦), T_3-achtige (●) en T_4-achtige BDE (▲) in de T-screen en hun chemische structuren.

De T-screen blijkt een goed bruikbare test om stoffen op te sporen die het functioneren van de TR beïnvloeden. Een beperking van de T-screen is echter dat deze geen effecten kan opsporen die via de verschillende TR's verlopen (α en β). Deze twee vormen van TR's spelen een eigen rol in het lichaam en stellen verschillende eisen aan de moleculen die ze binden. De T-screen blijkt wel een bruikbare stap in het screenen van stoffen die mogelijk effecten veroorzaken die verlopen via de TR's. Deze screening moet ook plaats vinden in aanwezigheid van T_3 om stimulerende stoffen zoals de T_3-achtige BDE, remmende stoffen zoals BDE209 en amiodarone of versterkende stoffen zoals hexabromocyclododecane (HBCD) te kunnen bepalen.

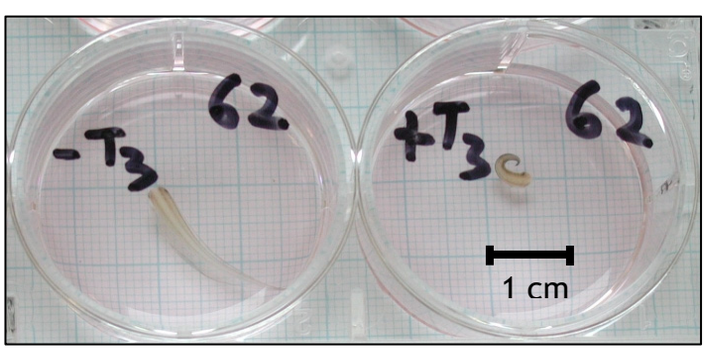
2. TRα/β specifieke reporter gen test (hoofdstuk 3)

Omdat de twee verschillende thyroïdhormoon receptoren (TR's) een belangrijke rol spelen bij de weefsel specifieke effecten van T_3, werd de TR-specificiteit van een aantal stoffen onderzocht die eerder actief bleken in de T-screen. Hiertoe werd een apen nieren cellijn (CV-1) voorzien van kikker (Zuid Arikaanse klauwpad, *Xenopus laevis*) TRα of TRβ plus een luciferase reporter gen. Wanneer

een stof een specifieke TR bindt, zal luciferase worden gevormd. Dit eiwit komt normaal voor bij vuurvliegjes (*Photinus pyralus*) en is eenvoudig te meten doordat het in aanwezigheid van het substraat luciferine licht uitzendt. De TRα/β specifieke reporter gen test is een belangrijke methode om weefsel- of levensstadium specifieke effecten van hormoonverstorende stoffen te kunnen voorspellen/verklaren voor de *in vivo* situatie. Deze test is een geschikte aanvulling op het testen van stoffen die actief zijn in de T-screen.

3. *Ex vivo* staartjes test (hoofdstuk 4)

Om na te gaan of de *in vitro* aangetoonde effecten van stoffen ook in een echt weefsel van een dier optreden, werd een proef uitgevoerd met losse staartjes van *Xenopus laevis* kikkervisjes in kweekmedium. Deze staartjes verdwijnen langzaam onder invloed van T_3 in het kweekmedium (Figuur 3). De afname in lengte van de staartjes is een maat voor de schildklierhormoon activiteit van stoffen. Bij toevoeging van 20 nM T_3 zijn de staartjes na 3,5 dag nog maar de helft van hun originele lengte. Dit effect werd versneld door HBCD en juist vertraagd door BDE206.



Figuur 3. Uitvoering van de *ex vivo* staartjes test. Links zonder T_3, rechts na toevoeging van 1 nM T_3 in het kweekmedium.

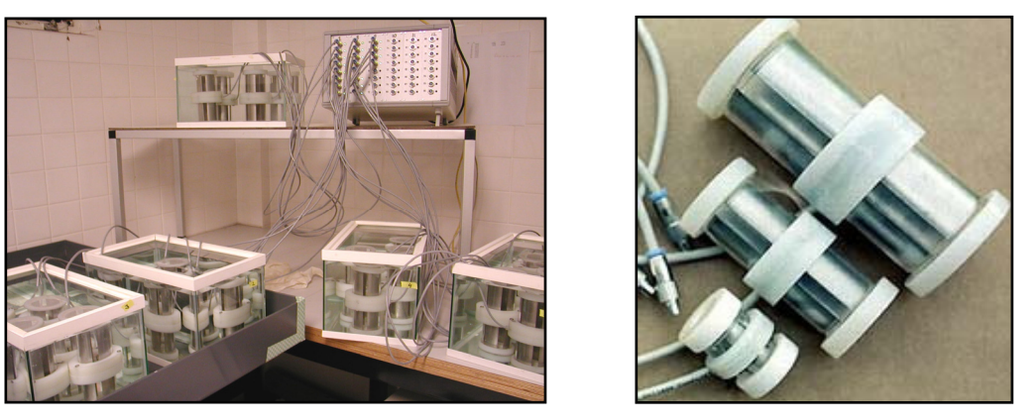
4. *In vivo* proliferatie test (hoofdstuk 5)

Om te onderzoeken of de effecten van HBCD en BDE206, ook in levende dieren (*in vivo*) optreden werden 1-week oude *Xenopus laevis* kikkervisjes blootgesteld aan dezelfde stoffen. Net als bij de T-screen werd eerst bepaald in hoeverre de stoffen het groeien van bepaalde cellen konden versnellen (ook wel proliferatie

genoemd) of vertragen. De effecten van HBCD en BDE206, al dan niet gecombineerd met T_3, werden bestudeerd in de hersenen en de voorzijde van de kop. De effecten die werden waargenomen waren weefsel-specifiek, wat te verwachten was door de totaal verschillende molekuulstructuren van de getestte stoffen. Zowel de *in vivo* proliferatie test en de *ex vivo* staartjes test bleken geschikt om de realiteitswaarde van eerdere *in vitro* resultaten te bewijzen en om de TR-specifieke effecten in meer detail te bestuderen. Ze zijn echter zeer tijdrovend en kosten bovendien veel proefdieren. Daarom zijn ze niet geschikt voor het snel testen van grote aantallen stoffen.

5. Multispecies Freshwater Biomonitor (MFB) (hoofdstuk 6)

De zogenoemde *Multispecies Freshwater Biomonitor* (MFB) (Figuur 4) werd toegepast om het gedrag van *Xenopus laevis* kikkervisjes te meten. Speciale meetkamertjes (Figuur 4) met elk 1 kikkervisje werden ondergedompeld in water met een bepaalde concentratie van de pesticide triphenyltin (TPT). De MFB kon automatisch verschillende soorten gedrag, zoals zwemmen, ademhaling en staartbeweging automatisch registreren. Blootstelling van *Xenopus laevis* kikkervisjes aan TPT bleek met name met het zwemgedrag van kikkervisjes te verminder. De resultaten tonen de geschiktheid van de MFB aan als nieuwe testmethode voor de automatische registratie van effecten van stoffen op het gedrag van *Xenopus laevis* kikkervisjes.



Figuur 4. Opstelling van de Multispecies Freshwater Biomonitor (MFB) (links) en foto van de meetkamertjes.

Mechanistische inzichten

Uit de combinatie van de verschillende testuitkomsten zoals beschreven in dit proefschrift, blijkt duidelijk dat stoffen de werking van T_3 kunnen remmen of versterken via verschillende TR's. Alleen stoffen die zeer sterk lijken op T_3, zoals de T_3- en T_4-achtige BDE, zijn in staat de werking van T_3 te imiteren. Andere stoffen, zoals BDE206, remmen de werking van T_3 via de TR. Omdat het HBCD-molecuul op geen enkele manier lijkt op het T_3-molecuul, en de stof ook niets deed in de TR-specifieke reporter gen test, is het onwaarschijnlijk dat de T_3-versterkende werking van HBCD via directe TR-activatie verloopt.

Gevoeligheid van de nieuwe testen voor T_3

De T-screen, TRα/β specifieke reporter gen test, *ex vivo* staartjes test en *in vivo* proliferatie test variëeren in gevoeligheid voor T_3. Dit zou verklaard kunnen worden door verschillen in snelheid waarmee de stoffen de TR's bereiken in een test met een enkele laag cellen (*in vitro*), in echte weefsels (*ex vivo*) of in kleine kikkervisjes met een actieve bloedsomloop (*in vivo*).

De testen zoals beschreven in dit proefschrift zijn veelbelovend om *in vitro*, *ex vivo* en *in vivo* de effecten van stoffen op de werking van T_3 te kunnen meten. De *in vitro* effecten zijn sterk voorspellend voor de *in vivo* effecten. Het is van belang dat stoffen ook getest worden in aanwezigheid van T_3, omdat in de i*n vivo* situatie ook een fysiologische concentratie T_3 aanwezig is en sommige stoffen pas dan hun hormoon verstorende werking laten zien.

Referenties

Brouwer, A., Morse, D.C. et al., 1998. **Toxicol Ind Health 14**, 59-84.
Schreiber, A.M., Das, B. et al., 2001. **P Natl Acad Sci USA 98**, 10739-10744.
Weber, R., 1967. Biochemistry of amphibian metamorphosis. Weber R., editor. NY: academic press.
Wit, C. de, Jansson, B., 1992. **Chemosphere 25**, 185-188.
Zoeller, R.T., 2005. **Mol Cell Endocrinol 242**, 10-15.

Dankwoord

Zeer toepasselijk voor dit proefschrift kan het AIO-schap vergeleken worden met de metamorfose van een kikkerembryo naar kikkervis en uiteindelijk kikkertje. Jaar 1 kenmerkt zich door de embryonale fase, waarin de AIO zich nog nauwelijks bewust is van zijn eigen (vijandig) extern klimaat. De eerste spierstrekkingen (schrijfpogingen) zijn waarneembaar, maar de veilige dooierzak wordt niet verlaten (de afdeling). In jaar 2, de premetamorfose, worden de eerste voorzichtige zwembewegingen ondernomen en vindt daardoor verkenning plaats van de omgeving (bezoek van congressen). In het derde jaar, de prometamorfose, breken de achterpootjes door (1e publicatie) en vindt sterke ontwikkeling plaats van neuraal weefsel (hersenen). Gedurende deze kritische fase vinden mogelijk interacties plaats met talloze gevaarlijke predatoren (boze reviewers ed.). Na deze fase bereidt de AIO zich langzaam voor op jaar 4, de metamorfe climax. In dat laatste belangrijke jaar komen de voor- en achterpoten helemaal tot ontwikkeling (acceptatie nieuwe artikelen en afronding proefschrift) en verdwijnt het staartje (laatste spoortje naïviteit). Nadat het kersverse kikkertje (doctor) zijn eerste vliegje naar binnen heeft gewerkt (promotiebuffet) is het dan nu toch tijd geworden om de uitdagende landfase (nieuwe baan) definitief te betreden, waar weer nieuwe gevaren loeren (?).

In tegenstelling tot de metamorfose van een kikker staat of valt een AIO-schap met een goede parentale zorg (begeleiding) en voldoende morele ondersteuning. Dit proefschrift was ook zeker niet tot stand gekomen zonder de bijdrage van talloze mensen die ik hierbij graag wil bedanken. Allereerst wil ik mijn co-promotoren, Tinka Murk en Dave Furlow, bedanken voor hun stimulerende begeleiding.

Tinka, bedankt voor die geweldige kans die je me gegeven hebt om onder jouw hoede te mogen promoveren. Ik ben ervan overtuigd dat ons beider enthousiasme, koppigheid en doorzettingsvermogen geleid heeft tot een prachtige synthese, die dit proefschrift zeker ten goede is gekomen. Ik denk met

heel veel plezier terug aan ons bezoek in Wenen (zes uur), Highway 1 richting Davis met zijn vele bochtjes (buhhh) en ons laatste overleg in de "nectarbar".

Dave, your lab including your excellent supervision played a crucial role in our understanding of the many aspects that involve thyroid hormone disruption. I am grateful for the opportunities that you created, and honored having you in Wageningen on the day of my PhD-defense.

Mijn promotor Ivonne Rietjens wil ik graag bedanken voor haar goede adviezen en vermogen om de spanningsboog altijd strak te houden. Jouw "zowel woorden als daden" mentaliteit was erg leerzaam en heeft in alle opzichten bijgedragen aan het tot stand komen van mijn proefschrift.

Bas Blaauboer, bedankt voor die leuke POT-opdracht in de periode tussen het einde van mijn AIO-contract en het ter perse gaan van mijn proefschrift. Het gaat vast een prachtige CD worden!

Uiteraard ben ik mijn (oud)-collega's van Toxicologie veel dank verschuldigd voor de goede sfeer op de afdeling en de vele gezellige momenten: Hans, Bert, Maaike, Anne-Marie, Annemarie, Ans, Jac, Walter (alias Gűnther), Pim, Ans., Elton, Irene, Gerrit, Gré, Wiratno, Letty, Vincent^2, Ashwin, Yee, Suzanne, Arno (dr^2), Aukje, Jelmer, Hester, Marjan, Yvonne, dhr. Zaagmans en last but not least mijn lievelingsmeisjes Laura en Marelle. Ik heb altijd met plezier met jullie samengewerkt en koester de vele prettige herinneringen zoals vakgroepuitjes, borrels, Sinterklaas en de Kerstlunch. Enne, die appeltaart komt nog wel een keer!

Frank, Timo en Eric, bedankt voor jullie vriendschap en eeuwige morele/professionele steun!!! Mijn oud-studenten, Elton, Cozmina, Martin, Els, Joost en Melissa, ben ik zeer dankbaar voor hun bijdrage aan mijn onderzoek. Het klinkt als een cliché, maar ik hoop dat jullie net zoveel van mij geleerd hebben als ik van jullie! Heel veel succes met jullie verdere carrière. Een speciaal woordje van dank gaat uit naar mijn paranimfen. Marcel, met jou als kamergenoot hebben we samen onze tijden van voor- en tegenspoed beleefd. In tijden van tegenspoed, wisten we elkaar echter op gepaste wijze weer op te monteren, waardoor ik altijd met plezier naar de afdeling ging. Ik zal onze diepgaande (semi)-

wetenschappelijke discussies nooit vergeten en het is goed om te weten dat jou bij Organon een mooie carrière wacht. Joost, als studenten stonden wij samen aan de basis van dit proefschrift. Ik vind het fantastisch dat wij in de loop van de jaren beste vrienden gebleven zijn en ben vereerd dat jij als paranimf naast mij op het podium zitting wilt nemen.

Lieve vrienden, uiteraard bestaat het leven uit veel meer dingen dan onderzoek doen en daarvan hebben jullie mij de laatste jaren prima doordrongen gehouden. Op de eerste plaats dank aan mijn hechte vriendengroep, de heeren Bart, Gerlof, Geert, Eric, Timo, Joost, Marco, Juul en Jorke en hun partners met een speciaal woordje van dank aan Marieke. We maken d'r een leuke dag van en uiteraard stimuleer ik jullie om je tijdens het feest op alle mogelijke manieren te wreken op mijn acties van de afgelopen jaren. Eric N. and Lenore, thanks for your friendship during my stay in Davis. I will always remember the funny moments that we experienced in the US, and I sincerely hope that we will meet again. Hoogstraat-buurtjes (Harro, Sjoerd et al), bedankt voor jullie wijze levenslessen. Het is zeer de vraag wie nu de meester en wie de leerling is. Geert, Barry en Fleurtje, bedankt dat ik als paranimf bij jullie een beetje mocht oefenen op het podium...nu is het mijn beurt! Mijn (oud)-huisgenoten ben ik dankbaar voor de gezelligheid, excellente kookkunsten en nog belangrijker hun flexibiliteit om deze soms op mijn (werk)schema aan te passen. Lieve Petra, dankzij jou geloof ik niet meer in toeval! Lot en Liek, als kinderen maakten we het elkaar niet altijd even makkelijk ("de koning slaapt"), maar mijns inziens heeft dit onze banden gesterkt zodat ik jullie nu mag rekenen tot mijn allerliefste vriendinnen. Dirk (white rastaman), je bent een geweldige aanwinst voor ons gezin, maar hoe zat dat nu met die hangmat? Kosta, please take excellent care of my sister, but I am completely sure you'll do so. Opa, ik voel me vereerd om u als oudste "gezinslid" erbij te mogen hebben en heb groot respect voor uw doorzettings- en ondernemingsvermogen. Tenslotte, lieve Pap en Mam, mijn dank aan jullie is nauwelijks in woorden uit te drukken maar de successen die Lotte, Lieke en ik op dit moment en in de toekomst mogen oogsten zijn te danken aan jullie "warme nest", centraliserende spil, zorg,

vertrouwen en altijd aanwezige liefde. Pap, onze vriendschap en jouw steun is van essentieel belang geweest voor het tot stand van mijn proefschrift. Laten we schouder aan schouder verdergaan in het leven en nog vele nieuwe (spannende) avonturen tegemoet gaan! Enne, het komt goed!

Curriculum Vitae

Merijn Schriks werd op 29 april 1976 geboren in het St. Annaziekenhuis te Geldrop. In 1993 voltooide hij de St. Radboud MAVO te Asten, waarna hij aansluitend startte met een opleiding aan het Middelbaar Laboratorium Onderwijs (MLO) te Eindhoven met als specialisatie analytische chemie. Na een orienterende stage op het laboratorium van de Centrale Suiker Maatschappij (CSM) te Breda, sloot hij in 1998 deze opleiding af met een afstudeerstage van zes maanden bij NOTOX B.V. te 's-Hertogenbosch, onder begeleiding van Richard Luit en Rinus Bogers. In datzelfde jaar begon hij aan de opleiding Milieuanalyse aan de Internationale Hogeschool Larenstein te Velp. Zijn orienterende stage deed hij onder begeleiding van dr. Marius Claassen bij de CSIR te Pretoria (Zuid-Afrika). Hij sloot zijn studie in 2001 af met een stage van zes maanden bij de afdeling Toxicologie aan Wageningen Universiteit onder begeleiding van dr. Arno Gutleb en dr. Tinka Murk. Aansluitend startte hij zijn promotie-onderzoek aan deze afdeling, waarvan de resultaten in dit proefschrift beschreven zijn. Een deel van de resultaten werden behaald gedurende een drie maanden durende onderzoeksperiode aan de University of California, Davis (VS) onder begeleiding van dr. J. David Furlow. Sinds 15 augustus 2006 is hij werkzaam bij Royal Haskoning, divisie Environment (adviesgroep Chemicals Management) te Nijmegen.

Merijn Schriks was born on the 29th of April 1976 in the St. Anna hospital in Geldrop. He finished his secondary education at the St. Radboud MAVO in Asten in 1993 after which he started a degree at MLO (intermediate laboratory education) in Eindhoven specializing in analytical chemistry. After an internship with the laboratory of CSM, the central sugar company in Breda, he finished his degree with a six-month internship with NOTOX B.V. in 's-Hertogenbosch, supervised by Richard Luit and Rinus Bogers. In the same year he started his degree in Environmental Analysis at the Internationale Hogeschool Larenstein in Velp. He took an internship with the CSIR in Pretoria (South-Africa) under the supervision of dr. Marius Claassen. He finished his degree in 2001 with a six-month internship at the department of Toxicology of Wageningen University where he was supervised by dr. Arno Gutleb and dr. Tinka Murk. He proceeded with his PhD research in this department, the results of which are described in this thesis. Part of his PhD research was conducted during the three months that he spent at the University of California, Davis (US) where he was supervised by dr. J. David Furlow. Since the 15th of August 2006 Merijn holds a job at the Environment Division of Royal Haskoning (adviesgroep Chemicals Management) in Nijmegen.

List of publications

Merijn Schriks, Elton Zvinavashe, J. David Furlow, Albertinka J. Murk (2006). Disruption of thyroid hormone-mediated *Xenopus laevis* tadpole tail tip regression by hexabromocyclododecane (HBCD) and 2,2',3,3',4,4',5,5',6-nona brominated diphenyl ether (BDE206). **Chemosphere, in press**.

Merijn Schriks, Melissa K. van Hoorn, Elisabeth J. Faassen, Joost W. van Dam, Albertinka J. Murk (2005). Real-time automated measurement of *Xenopus laevis* tadpole behavior and behavioral responses following triphenyltin exposure using the Multispecies Freshwater Biomonitor (MFB). **Aquatic Toxicology 77**, 298-305.

Merijn Schriks, Cozmina M. Vrabie, Arno C. Gutleb, Elisabeth J. Faassen, Ivonne M.C.M. Rietjens, Albertinka J. Murk (2005). T-screen to quantify functional potentiating, antagonistic and thyroid hormone-like activities of polyhalogenated aromatic hydrocarbons (PHAHs). **Toxicology in Vitro 20**, 490-498.

Arno C. Gutleb, Ilonka A.T.M. Meerts, Joost H. Bergsma, Merijn Schriks, Albertinka J. Murk (2005). T-screen as a tool to identify thyroid hormone receptor active compounds. **Environ Toxicol Pharmacol 19**, 231-238.

Merijn Schriks, Julie M. Roessig, Albertinka J. Murk, J. David Furlow (2006). Thyroid hormone receptor isoform selectivity of thyroid hormone disrupting compounds quantified with an *in vitro* reporter gene assay. (***Submitted*).**

Merijn Schriks, Eric S. Neff, Ivonne M.C.M. Rietjens, Albertinka J. Murk, J. David Furlow (2005). *In vivo* effects of nona brominated diphenyl ether (BDE206) and hexabromocyclododecane (HBCD) on cell proliferation in *Xenopus laevis* tadpoles: comparison with an *in vitro* cell based assay. (***Manuscript in preparation).***

Arno C. Gutleb, Merijn Schriks, Leonie Mossink, Hans J.H. van den Berg, Albertinka J. Murk (2005). A synchronized amphibian metamorphosis assay as an improved tool to detect thyroid hormone disturbance by endocrine disruptors and apolar sediment extracts. ***(Submitted).***

Arno C. Gutleb, Leonie Mossink, Merijn Schriks, Hans J.H. van den Berg, Albertinka J. Murk (2005). The prolonged-FETAX detects effects of exposure to environmentally relevant concenentrations of POPs that are not detected in the FETAX. ***(Submitted).***

Abstracts

Merijn Schriks, Cozmina M. Vrabie, J. David Furlow, Albertinka J. Murk (2006). Influence of Polyhalogenated Aromatic Hydrocarbons (PHAHs) on thyroid hormone-mediated cell response *in vitro*. **Society of Enviromental Toxicology and Chemistry (SETAC), 16th meeting in The Hague (The Netherlands)**.

Merijn Schriks, Eric S. Neff, Albertinka J. Murk, J. David Furlow (2006). Antagonistic and potentiating effects of BDE206 and hexabromocyclododecane (HBCD) in a functional *in vivo* assay with *Xenopus laevis* tadpoles. **Society of Enviromental Toxicology and Chemistry (SETAC), 16th meeting in The Hague (The Netherlands).**

Merijn Schriks, Cozmina M. Vrabie, J. David Furlow, Albertinka J. Murk (2005). Influence of Polyhalogenated Aromatic Hydrocarbons (PHAHs) on thyroid hormone mediated cell responses *in vitro* and *ex vivo*. **Endo 2005, 87th meeting (the forum on Endocrine Disrupting Chemicals) in San Diego (USA).**

Merijn Schriks, Ivonne M.C.M. Rietjens, Albertinka J. Murk (2004). Development and application of amphibian based testsystems for putative thyroid hormone disruption by Poly Halogenated Aromatic Hydrocarbons (PHAHs). **Netherlands Society of Toxicology (NVT), Veldhoven (The Netherlands).**

Merijn Schriks, Arno C. Gutleb, Albertinka J. Murk (2002). Thyroidogenic potency of compounds determined with the T-screen (2002). **Society of Environmental Toxicology and Chemistry (SETAC), 12th meeting in Vienna (Austria).**

Merijn Schriks, Ivonne M.C.M Rietjens, Albertinka J. Murk. Effects of Poly Halogenated Aromatic Hydrocarbons (PHAHs) on the development and metamorphosis of amphibians. Development of biomarkers and ecological relevant effects (2001). **Netherlands Society of Toxicology (NVT), Nijmegen (The Netherlands).**

Training and Supervision Plan
(Overview of conferences and courses attended during PhD)

2006:	NVT symposium, Wageningen (NL)
	SETAC conference, The Hague (NL)
2005:	Organ toxicology (PET)
	A1 Environmental research in context (SENSE)
	A2 Research context activity (SENSE)
	Career development (NWO)
	ENDO conference, San Diego (USA)
2004:	Pathobiology (PET)
	Epidemiology (PET)
	NVT PhD symposium, Veldhoven (NL)
	Reproduction Toxicology (PET)
2003:	Medical and forensic Toxicology (PET)
	Behavior and hormones (WUR)
2002:	Laboratory animal science (PET)
	Special topics in ecotoxicology (SENSE)
	SETAC conference, Vienna (AUSTRIA)
	Organising and coaching M.Sc-students (WUR)
2001:	Techniques for writing and presenting a scientific paper (Mansholt)
	NVT PhD symposium, Nijmegen (NL)
	Environmental and occupational toxicology (WUR)

The studies in this thesis were financially supported by a CEFIC-EMSG grant (project coordinator Christ de Rooij).

Publication of this thesis was financially supported by Wageningen University, Wageningen, The Netherlands.

Cover: Picture of a one-week old *Xenopus laevis* tadpole, exposed to 1 nM T_3 (back) and DMSO-solvent (front). Proliferating cells (mitotic phase) were labeled with a fluorescent antibody against the phospho-histone H3 mitosis marker and visualized with a Leica MZ FLIII fluorescence stereo dissection microscope equipped with a GFP2 filter set (a 480/40-nm excitation filter and a 510-nm barrier filter). Photographs were made with an Optronics LE750 digital camera (chapter 5).

Design cover: Koen Corstens

Printed by Ponsen & Looijen B.V. , Wageningen, The Netherlands