# Detecting the effects of environmentally relevant concentrations of thyroid hormone disrupting compounds on amphibian development

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"The flourishing of human and non-human life on Earth has intrinsic value. The value of non-human life forms is independent of the usefulness these may have for narrow human purposes".

(Arne Naess, 1989)

## Abstract

### Detecting the effects of environmentally relevant concentrations of thyroid hormone disrupting compounds on amphibian development

Persistent organic pollutants such as PCBs have been hypothesized to contribute to the observed global decline of amphibian populations. Thyroid hormone (TH) disruption is one of the possible mechanisms for effects of xenobiotics on amphibian development. In addition to the important functions shared with other vertebrates, TH also has an important function in amphibians. The metamorphosis of amphibian larvae to froglet needs a TH surge shortly before the onset of metamorphosis to proceed. To study the potential disruption during two specific life-stages a bioassay with exposure during very early developmental stages and one for exposure of tadpoles just before the onset of TH dependent metamorphosis were developed. The assays were optimized, validated with PCBs as standards and applied for testing of realistic dosages of polluted sediment extracts. In addition, an *in vitro* assay using the TH dependent growth of a cell-line was established as a screening tool.

The studies presented in this thesis reveal that the currently used early life stage test (FETAX) does not detect effects of the tested PCBs and apolar sediment extracts during the 96-hour test period whereas the newly developed prolonged-FETAX showed significant delayed effects of very low exposure concentrations on body weight, and on the period until- and percentage of animals finishing successful metamorphosis.

In the so-called Synchronized Amphibian Metamorphosis Assay thiourea was used to synchronize tadpole development in NF stage 54 thereby starting experiments with a very homogenous group of animals. Significant effects of oral exposure to PCBs and apolar sediment extracts were found on the period until metamorphosis, and the distribution of the developmental stages of tadpoles that did not finish metamorphosis.

The approach with exposing embryos (prolonged-FETAX) and tadpoles (Synchronized Amphibian Metamorphosis Assay) may finally better reflect environmental risks of apolar compounds than limiting the exposure to solely a single life-stage or water-exposure.

The effects of the highly diluted apolar sediment extracts suggest the presence of TH disrupting compounds in the aquatic environment and possible effects of such compounds on development of amphibians and possibly other aquatic vertebrate species in the wild. The *in vitro* model showed its suitability and importance to study specific aspects of endocrine disrupting potency of toxic compounds.

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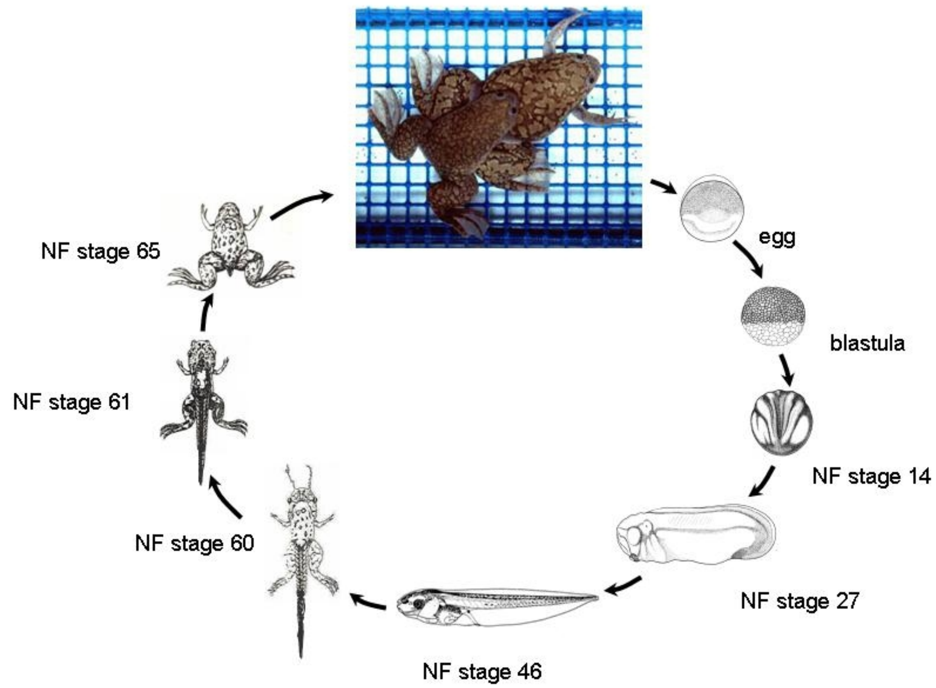
## Abbreviations

| | |
|---|---|
| 2,3,7,8-TCDD | 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin |
| ACTH | adrenocorticotropic hormone |
| AhR | aryl hydrocarbon receptor |
| DMSO | dimethylsulfoxide |
| DPH | 5,5′-diphenylhydantoin |
| EDCs | endocrine disrupting compounds |
| EROD | ethoxy resorufin *O*-deethylase |
| FETAX | Frog Embryo Teratogenesis Assay-*Xenopus* |
| MMI | 2-mercapto-1-methyl-imidazole |
| p,p′-DDT | 4,4'-(2,2,2-trichloroethane-1,1-diyl)bis(chlorobenzene) |
| PCBs | polychlorinated biphenyls |
| PHAHs | polyhalogenated aromatic hydrocarbons |
| POPs | persistent organic pollutants |
| PROD | pentoxy resorufin *O*-deethylase |
| SULTs | sulfotransferases |
| $T_3$ | 3,3′,5-triiodothyronine |
| $T_4$ | 3,3′,5,5′-tetrathyroxine |
| TBG | thyroxine-binding-globulin |
| TEF | toxic equivalency factor |
| Tetrac | 3,3′,5,5′-tetraiodothyroacetic acid |
| Tg | thyroglobulin |
| TH | thyroid hormone(s) |
| TR | thyroid hormone receptor |
| TRH | thyrotropin-releasing hormone |
| Triac | 3,3′-5-triiodothyroacetic acid |
| TSH | thyroid-stimulating hormone |
| TTR | transthyretin |
| TU | thiourea |

# 1

# General Introduction




Life cycle of *Xenopus laevis*

# Detecting the effects of environmentally relevant concentrations of thyroid hormone disrupting compounds on amphibian development

## Introduction

The research presented in this thesis concerns low dose effects of thyroid hormone disrupting compounds on the development of amphibian larvae exposed during sensitive periods. The motive for this research were the reports of amphibian declines occurring worldwide even in what seemed to be undisturbed areas such as within the borders of national parks (Blaustein, 1994; Pechman and Wilbur, 1994; Dalton, 2000; Alford et al., 2001; Stockstad, 2004; Stuart et al., 2004; Williams, 2004). In addition to increased UV irradiation, environmental contamination with persistent organic pollutants (POPs) was mentioned as one of the possible factors contributing to this decline (Carey and Bryant, 1995). POPs originating from atmospheric deposition have been shown to reach even the most remote areas (Hamers et al., 2003; Scheringer et al., 2004; Wethington and Hornbuckle, 2005).

Exposure to very low concentrations of hormone-mimicking compounds can disturb development and reproduction of man and wildlife (Carlsen et al., 1995; Skakkebaek, 2002; Nash et al., 2004; Ottinger et al., 2002). In addition to sex-steroid hormone disrupting compounds there may be also compounds present in the environment that disturb thyroid hormone homeostasis. Thyroid hormone (TH) is important in development and in many regulatory functions in vertebrate species (Porterfield and Hendrich, 1993;

Tata, 1999; Power et al., 2001; Gudernatsch, 1912; Kanamori and Brown, 1996). In amphibians there is an additional important function of TH, as amphibian larvae are not able to pass metamorphosis without a TH surge shortly before the onset of metamorphosis (Gudernatsch, 1912).

Thus, the question posed at the start of this project included the following. Are persistent man-made compounds likely to contribute to this decline of amphibian populations at concentrations that are environmentally relevant? As developing organisms are at a stage in their lives when small alterations could make a significant, life-long difference the question was more specifically: what stages of development are most sensitive for adverse effects upon exposure to realistic concentrations of thyroid hormone disrupting POPs. The focus is not on adult amphibians, as their organs and tissues are fully organized, feedback mechanisms keep physiological parameters within acceptable ranges and metabolism and excretion of toxic compounds usually is well developed. Embryos and tadpoles, however, are in the midst of this developmental process and during metamorphosis, the last phase of this development tadpoles are fully remodelled within a short period of time.

## The world of amphibians

Amphibians are intermediate in some way between fully aquatic fish and terrestrial reptiles. Amphibians have evolved in Mid-Devon, nearly 350 million years ago and exist in a wide range of morphological and ecological types with more than 3900 species currently known and new species still being described every year. Essentially amphibians can be defined as quadrupedal (four legged) vertebrates with glandular skin without epidermal structures (scales, feathers, hairs) characteristic for other groups of tetrapods

(birds, reptiles, mammals). They include frogs, toads, salamanders, newts, and caecilians. Amphibian eggs must develop in moist conditions, as their mucoid capsules are highly permeable. This quality makes the eggs vulnerable for desiccation and permeation of environmental contaminants (Duellman and Trueb, 1994).

The physiology of metamorphosis from aquatic tadpoles to usually terrestrial adults has been intensively studied and much of early knowledge on TH and pituitary hormones in vertebrates has come from endocrinological studies on amphibians (Gudernatsch, 1912). The ease of breeding of some amphibian species in the laboratory has made them valuable experimental animals for a wide range of scientific applications (Sparling et al., 2000).

Over the last 50 years, many species of amphibians throughout the world have declined markedly in numbers and some species have become extinct (Blaustein and Kiesecker, 2002; Houlahan et al., 2000; Wake, 1991; Young et al., 2001, Williams, 2004). In many instances, these declines are attributable to adverse human influences acting locally, such as deforestation, draining of wetlands, and pollution. About 15 years ago, however, herpetologists from practically all parts of the world started to report declines in amphibian populations in protected, apparently pristine habitats, such as national parks and nature reserves (Wake, 1991; Blaustein, 1994; Pechman and Wilbur, 1994). This led to the suggestion that in addition to local causes there may be one or more global factors that are adversely affecting amphibians. Possible candidates for such influences are climatic and atmospheric changes, such as increased UV-B radiation, diseases and widespread pollution, including acid rain, and disease (Kiesecker et al., 2001). Thus, it was suggested that wildlife populations are affected critically by sublethal impacts of anthropogenic disturbances. Yet little research has focused on such effects on amphibians.

## Endocrine disrupting compounds (EDCs)

The presence of chemicals in the environment with the potential to disrupt endocrine systems, so-called endocrine disrupting compounds (EDCs), has become a major focus of research during the last years, as both wildlife and humans may be affected (Charles, 2004; Charles et al., 2005; Fisher, 2004; Tyler et al., 1998; Vos et al., 2000; Wester et al., 2004). The endocrine system presents a number of target sites for the induction of adverse effects by EDCs. Most of the attention focuses on (anti)estrogenic effects and test systems to determine potencies of compounds and extracts (Fujimoto and Honda, 2003; Hamers et al., 2003; Legler et al., 2000; Roepke et al., 2005; Safe, 2005; Sumpter, 1998). However, far less effort has been put in the identification of thyroid hormone disrupting compounds (Brouwer et al., 1998). In 2003 the three Validation Management Groups (VMGs) of the Organization for Economic Cooperation and Development (OECD) covering mammalian, ecotoxicity, and non-animal methods identified thyroid screening and testing as areas for further investigation by the OECD. The necessity to pay more attention to compounds that possibly interfere with this hormone system has been clearly formulated in recent years (EDSTAC, 1998; DeVito et al., 1999; Colborn, 2002).

## Thyroid hormone and EDCs

TH is important in development and in many regulatory functions in all vertebrate species (Porterfield and Hendrich, 1993; Power et al., 2001); but in amphibians there is an additional important function of TH. Without a TH surge shortly before the onset of metamorphosis, amphibian larvae are not able to successfully

metamorphose (Bray and Sicard, 1982; Galton, 1992; Mondou and Kaltenbach, 1979).

The mechanisms of action of EDCs (including thyroid hormone disrupting compounds) can be divided into:

- Disruption of the production, release, transport, or metabolism of natural hormones
- Agonistic/antagonistic effects ('hormone mimics')
- Disruption of the production and/or function of hormone receptors

Thyroid hormones (THs) are small biphenolic compounds derived from two tyrosine residues from a large protein – thyroglobulin (Tg), which is synthesized in the follicle cells of the thyroid gland. The iodinated Tg is stored in the colloid of the follicle lumen until it is required for the production of TH. After protolytic enzymes digest the iodinated Tg, 3,3′,5,5′-tetrathyroxine ($T_4$) and 3,3′,5-triiodothyronine ($T_3$) (Figure 1) are released and secreted into the plasma (Rang et al., 1995).

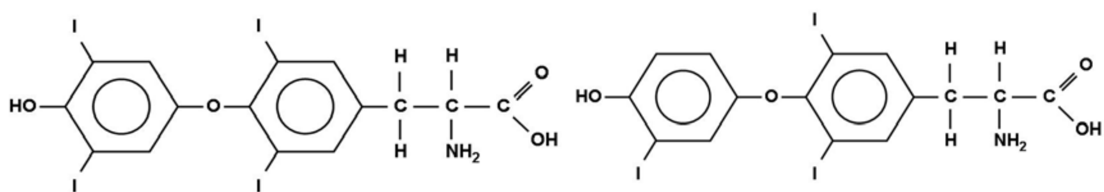



Figure 1. Molecular structures of $T_4$ (left) and $T_3$ (right)

$T_4$ and $T_3$ exert a negative feedback effect on the release of hypothalamic thyrotropin releasing hormone (TRH) and on the activity of pituitary thyrotropin stimulating hormone (TSH). TSH regulates the activity of the thyroid gland including synthesis and

release of THs, uptake of iodine, and even cell hypertrophy and hyperplasia (Spaulding, 2000). Thyroid hormones readily partition into cell membranes because of their lipophilicity. In the blood they are transported bound to specific proteins. In mammals $T_4$ is bound mainly to thyroxine-binding-globulin (TBG) or transthyretin (TTR) and the remainder to albumine. In amphibians TBG is not present (Larsson et al., 1985) and TTR preferentially binds $T_3$ instead of $T_4$, which is an inverse affinity compared to warm-blooded animals (Yamauchi et al., 1993).

$T_4$ and $T_3$ are actively transported into target tissues (Docter et al., 1997) after which $T_4$ can be converted to $T_3$ by the action of outer-ring deiodinases (Type I, II and III) with organ and cell-type specific distribution (Visser, 1996; Huang et al., 1999; St. Germain and Galton, 1997). Important pathways to regulate the hormone concentration are sulfotransferases (SULTs), which catalyze isozyme specific conversion of several ligands among which THs (Matsui and Homma, 1994; Visser et al., 1998). THs are cleared from the blood in the liver following glucuronidation by UDP-glucuronyl transferase (Hood and Klaassen 2000). The modified THs are then eliminated through the bile. Compounds can interfere on a cellular level with thyroid hormones by inhibiting the uptake or by competition with the receptor binding (Zemel et al., 1988; Topliss et al., 1989; Bogazzi et al., 2001) (Figure 2).

$T_4$ and $T_3$ are actively concentrated in target cells about 10 fold over that of the circulation, although this is tissue dependent. The receptors for $T_3$ (TRs) are nuclear proteins that bind to DNA and regulate transcription. There are two genes that encode the TRs, c-erbA-alpha (TRα) and c-erbA-beta (TRβ). Each of these genes is differentially spliced, forming separate TRs, TRα1 and TRα2 (a not $T_3$ binding form), TRβ1, TRβ2 and TRβ3. The relative abundance of these receptors in different tissues may contribute to the observed tissue-, cell-, and developmental stage-specificity of TH dependent

effects (Lazar, 1993; Oppenheimer and Schwartz, 1997; Sadow et al., 2002; Malm, 2004; Cheng, 2005a,b).

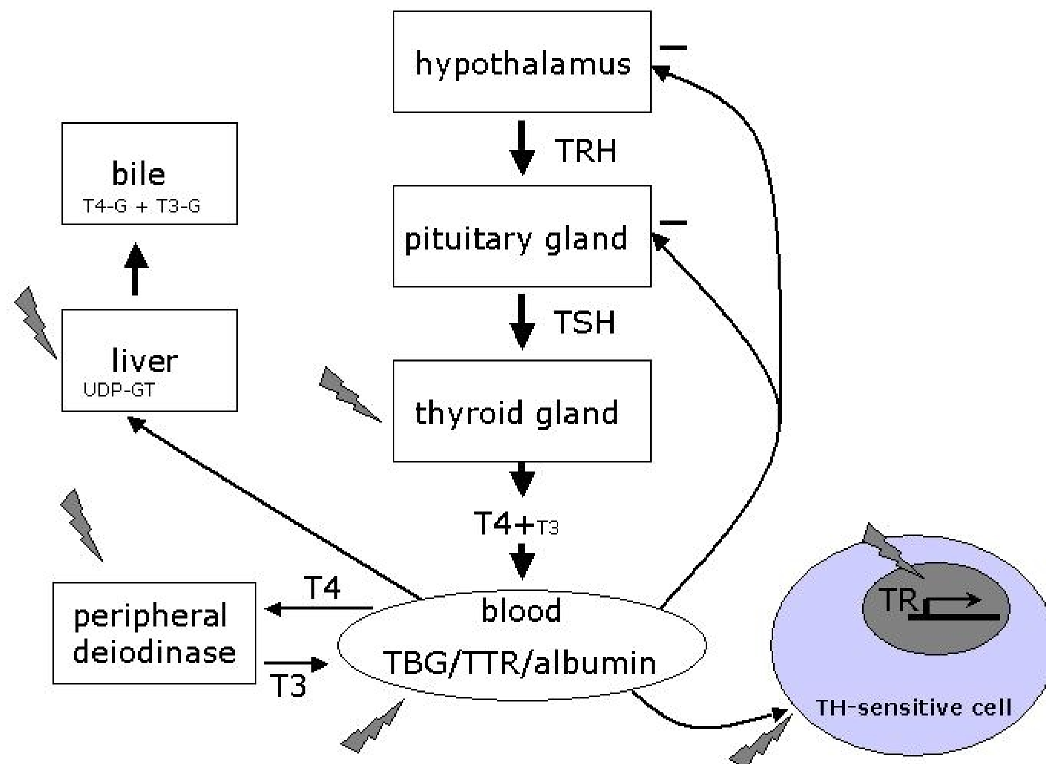



Figure 2. Important mechanisms for the regulation of TH concentrations and activity (based on Zoeller and Crofton, 2000).
Known sites of action of thyroid hormone disrupting compounds

EDCs reported to be able to disturb the TH system include the group of polyhalogenated aromatic hydrocarbons (PHAHs). For the group of PHAHs, probably the best-studied group of organic environmental contaminants, altered thyroid function and morphology were reported with ultra-structural lesions in thyroid follicular cells (Brouwer et al., 1989). Hydroxylated PHAHs, metabolites from the parent compounds show high binding affinity for TTR, which results in inhibition of $T_4$-binding to TTR and in addition in a disruption of the complex formed by TTR with the retinol binding protein (RBP) altering both plasma levels of TH and retinol in mammals (Brouwer and van den Berg, 1986; Lans et al., 1993; Murk et al., 1994; Meerts et al., 2000). Altered activity of TH metabolism mostly

accompanied by reduced plasma levels of $T_4$ were reported in PHAH exposed experimental animals. Tissue and enzyme specific activations and inhibitions of Type I- and Type II-deiodinases as well as of SULTs were induced by PHAHs resulting in altered activation of $T_4$ into $T_3$. Activation of the aryl-hydrocarbon receptor (AhR) is the main although not the sole mechanism of PHAH related toxicity (Safe, 1994). The induction of UDP-glucuronyl transferase by PHAHs and increased excretion of THs appeared to be under control of the AhR pathway so that a causal relationship with the PHAH exposure is very likely (for a review see Brouwer et al., 1998).

## The role of TH in amphibian development

Compared with other processes in vertebrate species amphibian development is perhaps most dependent on TH. Amphibian development consists of two phases, of which embryogenesis, development prior to uptake of food, occurs before the formation of a functional thyroid gland. Although all developmental stages following embryonic development are dependent on low TH concentrations, the timing of the metamorphosis – the transformation of a tadpole into a froglet, is absolutely dependent on the presence of a sufficient great TH surge (Bray and Sicard, 1982). TH exerts its role in early amphibian development mainly via the TRα and during metamorphosis via TRβ. Especially via the TRβ TH regulates the whole onset and completion of metamorphosis (Figure 3), including apoptosis of the tail and remodelling of tissues and organs (Sachs et al., 2000).
For different amphibian species staging tables have been developed of which the one for *Xenopus laevis* (Nieuwkoop and Faber, 1975) (later referred as NF stage) and *Rana sp.* (Gosner, 1960) are the

ones applied within this thesis. The first stages that are defined to be part of the metamorphosis are characterized by development of larval structures and body growth (early metamorphosis from NF stage 51 to NF stage 55), followed by development and growth of extremities (climax of metamorphosis, up to NF stage 60). This metamorphic development up to NF stage 60 is dependent on a surge in thyroid hormone concentration. The following stages up to completion of metamorphosis are characterised by remodelling of the body shape such as completion of the growth of extremities, tail resorption and decreasing thyroid hormone levels (late metamorphosis, up to NF stage 65). From NF stage 65 to NF stage 66 (the animals are froglets then) only the remains of the tail have to disappear, but this last part of the bodies remodelling is not TH dependent anymore.

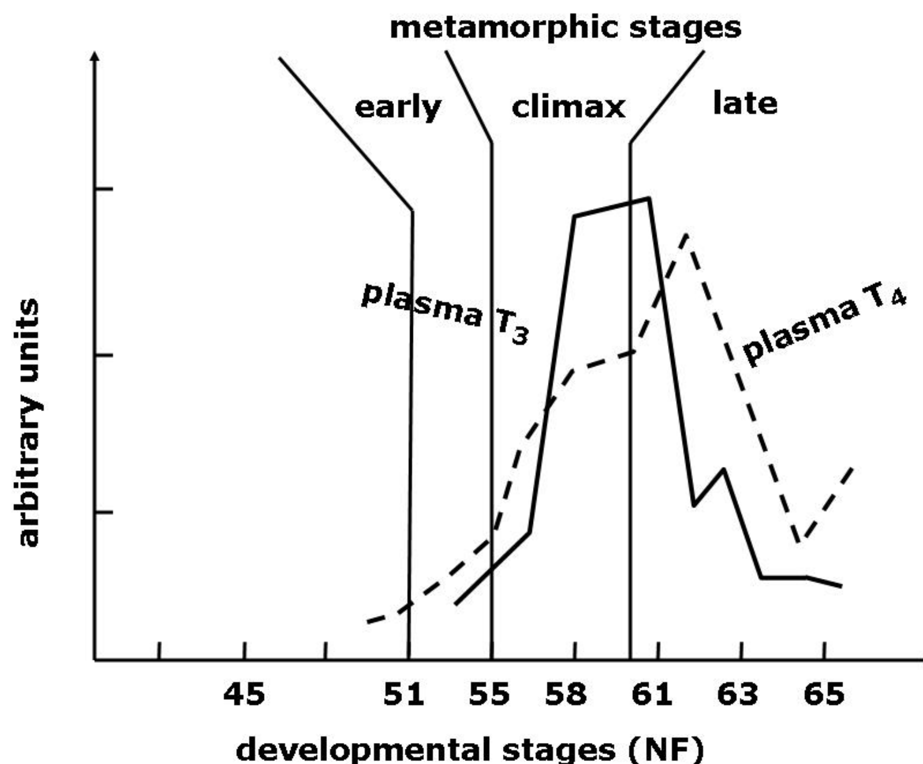



Figure 3. Changes in plasma TH levels in tadpoles of *Xenopus laevis* during the metamorphosis. Note the rise of $T_3$ and $T_4$ in stages following NF stage 54 (based on: Etkin, 1932; Bray and Sicard, 1982)

## Commonly performed bioassays to study effects of toxic compounds on amphibians

Amphibians are often used to study effects of toxic compounds on early development and thyroid hormone. The use of amphibians such as *Xenopus laevis* as a common test organism for teratogenicity is justified because the genetic programme controlling embryonic development is highly conserved over evolutionary time (Bantle, 1995). The bioassay most frequently performed with amphibians is the so-called Frog Embryo Teratogenesis Assay-Xenopus (FETAX), a short-term early-life time test covering the 4-day period of embryonic development of *Xenopus laevis*. The FETAX has been shown to be a powerful bioassay for the identification of compounds that exhibit acute embryotoxic potency (Bantle et al., 1994a,b; Bernardini et al., 1996; Dumont et al., 1983; Fort and Bantle, 1990; Fort and Paul, 2002; Fort et al., 1995, 2000b; Herkovits et al., 1997; Luo et al., 1993; Mann and Bidwell, 2000). In the FETAX three endpoints are applied, namely mortality, malformations and growth inhibition. The FETAX is also applied for aquatic toxicity assessments and for testing complex mixtures including industrial effluents and soil extracts (Fort et al., 1995). However, acute toxicity is an endpoint that does not include specific subtle physiological disturbances that may affect later stages of the complex development of amphibian tadpoles to terrestrial adults. Detecting subtle effects on metamorphosis ask for assays that cover more of the lifespan from embryo until froglet.

Examples of *in vivo* assays that have been proposed to study thyroid hormone disrupting compounds are 30-day limb development, 14-day tail resorption studies or a 28-day Xenopus Metamorphosis Assay (Fort et al., 2000a; Christensen et al., 2005; Opitz et al., 2005). All these assays are based on the conceptual

framework that TH disrupting compounds may enhance or reduce the rate of metamorphic development. These assays include morphological measures and differ in the exposure period and endpoints analysed.
The 30 days limb development assay follows embryo and tadpole development until hind limb development at NF stage 54 as the endpoint thereby covering mainly embryonic development and early metamorphosis whereas the thyroid hormone dependent climax of metamorphosis is not included (Fort et al., 2000a; Fort et al., 2004). Tail resorption in *Xenopus laevis* occurs from NF stage 58 to 66 when tail resorption is completed and this event is used in a 14-day test protocol that was applied to *Xenopus laevis* (Fort et al. 2000a) and to *Rana catesbeiana* (Christensen et al., 2005). However, this approach does not consider possible effects during early metamorphosis that may influence the metamorphic climax. In the 28-day Xenopus Metamorphosis Assay (XEMA) exposure starts at NF stage 48 to 50 with the developmental stage after 28 days of exposure as endpoint (Opitz et al., 2002, 2005). This protocol was recently also applied to *Silurana (Xenopus) tropicalis* (Mitsui et al., in press). In this assay the variation is relatively large because animals have to be used from three developmental stages and does not cover all events until complete amphibian metamorphosis.

**The choice of amphibian species used in bioassays**

*Xenopus laevis* is a widely used experimental animal that is easy to keep and relatively easy to breed with egg laying being inducible throughout the year. Using *Xenopus laevis* as a search keyword resulted in 18.876 counts in PubMed (search performed 27.8.2005). However in the few studies that compared the sensitivity of

*Xenopus laevis* with other amphibians, *Xenopus laevis* was found to be not always the most sensitive species for the compounds tested (Schuytema and Nebeker, 1998, 1999; Schuytema et al., 1991, 1995). At least with respect to its completely aquatic lifestyle *Xenopus laevis* is not representative for amphibians in general with the truly amphibian life traits of the frogs and toads of boreal climatic zones (North America, Europe and Asia). Amphibians are very diverse in all aspects of their biology, and it is not yet clear whether results from the tropical and phylogenetically primitive *Xenopus laevis* can be extrapolated to other amphibians such as the European common frog (*Rana temporaria*). The later species is living under temperate climatic conditions in Eurasia, showing the typical life trait for amphibians with aquatic larvae and terrestrial adults that reproduce in the water and representing a phylogenetically more modern frog. Much of the amphibian toxicological literature describes studies using representatives of the genera *Rana*, *Bufo* or Xenopus (Power et al., 1989; Mann and Bidwell, 1999, 2001) and as a result of these considerations *Xenopus laevis* and *Rana temporaria* were chosen as experimental animals in the present thesis.

## Outline of this thesis

The overall objective of the work described in this thesis was to study possible effects of relatively low doses of contaminants on amphibian development when animals are exposed during sensitive life stages. To this end two new in vivo bioassays with amphibian larvae were developed. One assay studying delayed effects of exposure during very early developmental stages, the other studying developmental effects of exposure just before the onset of thyroid hormone dependent metamorphosis. These assays were

optimized, characterized with PCB-standards and applied for testing of realistic dosages of polluted sediment extracts. In addition an in vitro assay to test the TH-mimicking potency of those contaminants was developed, characterized and applied.

In line with the objective, the thesis focuses on the following three research questions:

1) What are the most sensitive periods for disruption of amphibian development by toxic compounds?
2) Are effects to be expected at realistic exposure routes and concentrations?
3) What are the best methods to study and predict possible disruption of thyroid hormone dependent amphibian development?

The experiments performed to answer these questions are presented in this thesis as follows:

ad 1) Effects of exposure at different time points during amphibian reproduction and development were studied to identify sensitive periods either by dosing the egg via the female (chapter 2), by applying the FETAX-protocol of embryonic exposure and studying delayed effects in further unexposed development of the tadpoles (chapter 2 and 3), or by dosing tadpoles via their food either continuously (chapter 4 and 5) or through a 10-day exposure period (chapter 4). Experimental setup and most relevant exposure routes were chosen for tadpoles in that specific stage of development (Figure 4).

In addition, this thesis presents the results of an Intermezzo describing the results of an unintended observation of unexpectedly high toxicity for tadpoles of laboratory gloves initially used during

the experiments. Although not part of the answers to the scientific questions this information is relevant in order to protect life of experimental animals and to hinder bias of results due to unintended exposure.

ad 2) A range of environmentally relevant concentrations of either technical PCB mixtures resembling the congener pattern found in aquatic environments, single congeners (PCB 77 and PCB 126) and sediment extracts from polluted sites in the Netherlands were studied in the various newly developed and validated test protocols (chapter 2, 3, 4, 5,).

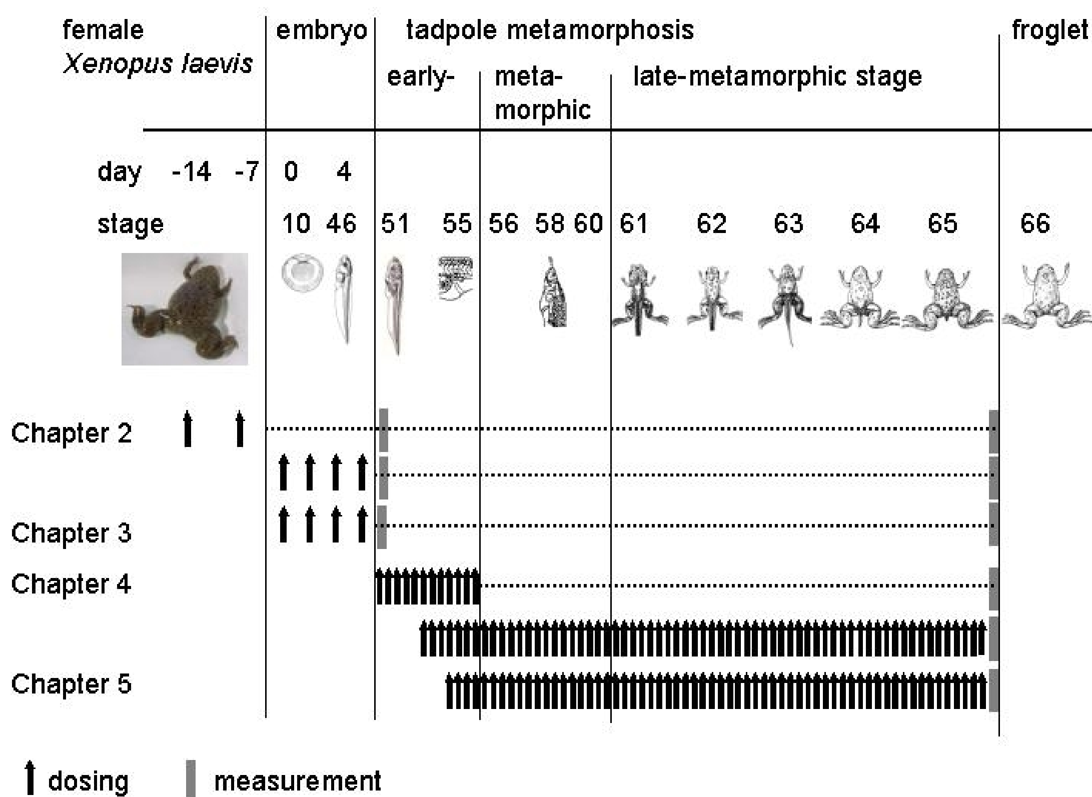



Figure 4. Time points of dosing and measurements as applied in the various experiments described in this thesis. Arrows indicate days on which animals were exposed, dotted lines indicate exposure-free intervals following the exposure period and grey bars indicate termination of experiments. Developmental stages (not on scale) according to Nieuwkoop and Faber (1975)

ad 3) In our search for the best method to study and predict possible disruption of thyroid hormone dependent amphibian

development two amphibian species, namely the South African clawed toad (*Xenopus laevis*) and the European common frog (*Rana temporaria*) were compared where applicable (chapter 2, 4 and Intermezzo).

To reduce the number of animals needed in order to be able to start with animals in exactly the same developmental stage, a synchronizing step was included in the protocol. By halting tadpole development in NF stage 54 using water spiked with the goitrogen thiourea, it is possible to overcome the problem of the rapid development of *Xenopus laevis* tadpoles in non-homogeneous test populations (chapter 5). In addition the initial relatively simple experimental setup (chapter 2 and 4) was replaced by a more sophisticated one in which water volume per animal was kept constant throughout the experimental to overcome the influence of mortality and metamorphosis on the density dependent development of the remaining tadpoles (chapter 3 and 5).

For reasons of replacement of animal experiments whenever possible an in-vitro assay based on thyroid hormone dependent cell growth of a rat pituitary cell-line (GH3) was established in order to test compounds and environmental extracts their TH mimicking potencies (chapter 6). The results of the in vitro tests are compared to the in vivo results.

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# 2

# Delayed effects of pre- and early-life time exposure to polychlorinated biphenyls (PCBs) on tadpoles of two amphibian species (*Xenopus laevis* and *Rana temporaria*)

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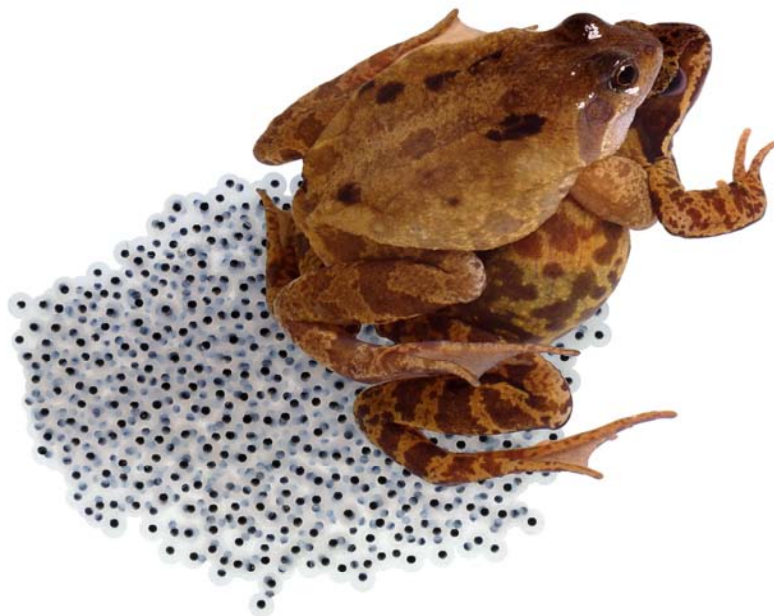

*Rana temporaria* in amplexus
(Photo courtesy of Jane Burton, http://www.warrenphotographic.co.uk)

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# Delayed effects of pre- and early-life time exposure to polychlorinated biphenyls on tadpoles of two amphibian species (*Xenopus laevis* and *Rana temporaria*)

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**Abstract**

This study examined the effects of polychlorinated biphenyls (PCBs) on the development of amphibians using *Xenopus laevis* and *Rana temporaria* as experimental animals. Amphibians were exposed at different life stages and via different routes to the technical mixtures Clophen A50 and Aroclor 1254 or to a non-ortho PCB congener (PCB 126). The effects of PCB exposure in amphibians, such as mortality, number and pattern of malformations, or body weight at the end of successful metamorphosis of tadpoles, depends on the route, the point of time of exposure during the complex life cycle of amphibians, and the length of the observation period. Retinoid concentrations were significantly altered in PCB dosed embryos. Presently used early-life time test systems such as the FETAX assay may underestimate toxic effects of compounds with long term response such as PCBs on amphibians. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Amphibian; Retinoids; Thyroid hormone; Metamorphosis; FETAX; PCBs

## 1. Introduction

Over the last decades a world-wide trend of decreasing amphibian populations in different types of habitats has been observed (Wake, 1991; Griffiths and Beebee, 1992; Blaustein, 1994; Pechmann and Wilbur, 1994). In addition to physical threats, such as habitat destruction and increased UV-radiation, ubiquitous environmental pollution with persistent toxic substances is suspected as one of the causative factors. Amphibians may be exposed to toxic compounds via several routes because of their semipermeable skin, the development of their eggs and gill breathing larvae in the water and their changing position in the food web from herbivorous tadpoles to carnivorous adults. Several publications have reported on their susceptibility to pollution and acidification of their environment (Freda and Dunson, 1985; Jung and Jagoe, 1995).

Polyhalogenated aromatic hydrocarbons (PHAHs) are known for their persistence, their plethora of toxic effects and enrichment in food chains in numerous areas and species (IPCS, 1993; Tanabe et al., 1994, Wania and Mackay, 1996). Gendron et al. (1997) and Leonards (1997) found evidence for PHAH accumulation in amphibians from the field.

The mechanisms of toxicity of PHAHs such as polychlorinated biphenyls (PCBs) are well investigated. In addition to the arylhydrocarbon receptor (AhR)-mediated induction of liver cytochrome P450 enzymes, PHAHs are able to disrupt metabolism and storage of retinoids (Zile, 1992) and thyroid hormone homeostasis (Brouwer et al., 1998). The parent compounds alter metabolism (Murk et al., 1994), whereas the hydroxylated metabolites disrupt the plasma transport of $T_4$ and all-*trans*-retinol (Brouwer and van den Berg, 1986).

It had been shown that PHAHs are passed from the females to eggs in amphibians (Jung and Walker, 1997)

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and may thus have direct effects on embryonic and early life stage development. In addition, PHAHs may indirectly affect embryonic development by altering vitamin A and thyroid hormone levels as a result of female exposure to PCBs and subsequent decreases in maternal blood levels of all-*trans*-retinol and thyroid hormone (Vieira et al., 1995a,b). Changes in the vitamin A pattern and reduced thyroid hormone levels in developing eggs of birds are known to be related with reduced temporal development or embryo mortality (Murk et al., 1996) even in late stages of development (Whittmann et al., 1993).

Thyroid hormone is crucial in embryonic development, growth and regulation of energy metabolism in all vertebrate species, as well as in the specific process of amphibian metamorphosis. Without a thyroid hormone surge shortly before the onset of metamorphosis amphibian larvae are not able to reach the post-metamorphic stage (Gudernatsch, 1912; Bray and Sicard, 1982). Thyroid hormone controls metamorphosis by regulating the expression of a cascade of genes (Shi, 1994) and decreased levels of thyroid hormones might result in prolonged metamorphosis, absence of metamorphosis or increased mortality of metamorphic larvae.

Early embryonic development is also strongly dependent on the presence of a specific gradient of active retinoids. All-*trans*-retinoic acid, all-*trans*-3,4-didehydro-retinoic acid, 9-*cis*-retinoic acid, and 14-hydroxy-4,14-retro-retinol are responsible for pattern formation, in particular for the orientation of tissue growth in the developing embryo (Pijnappel et al., 1993). Any change in the subtle balance of active retinoids may result in developmental abnormalities as it was shown for *Xenopus laevis* embryos after treatment with retinoic acid (Papalopulu et al., 1991).

The objectives of this study were to investigate the possible adverse effects of polychlorinated bipenyls (PCBs), a main chemical group within the PHAHs, on embryonic and larval development of amphibians. The African clawed-toad (*Xenopus laevis*), a species with solely an aquatic life style, a well observable and well described development (Nieuwkoop and Faber, 1975) and the common frog (*Rana temporaria*), a species representing the typical amphibian life history with a wide distribution in Europe, were chosen as experimental animals. Tadpoles were exposed to the technical PCB mixtures Clophen A50 or Aroclor 1254 or the dioxin-like non-ortho PCB congener 3,3′,4,4′,5-PeCB (PCB 126) either by dosing the females before their egg-laying period, or by exposing early stages of embryos via the water phase according to the standard test: Fetal Embryonic Teratogenic Assay using Xenopus (FETAX). In addition, possible delayed effects on the development of *X. laevis* were studied with the newly developed prolonged-FETAX assay. Retinoid and thyroid hormone concentrations were analysed at different developmental stages, and malformations of embryos and tadpoles, duration of the metamorphic transformation and weight after successful metamorphosis were followed throughout the experiments.

## 2. Animals, materials and methods

### 2.1. Animals, breeding and housing

All experiments were performed in the laboratory at the Toxicology Group, Wageningen University of Agriculture and Research Centre (WUR). Both amphibian species were kept at a 12:12 h photoperiod schedule. Adult *X. laevis* were obtained from the Department for Experimental Zoology, Catholic University of Nijmegen, The Netherlands. Adults were maintained in aquaria ($150 \times 60 \times 50$ cm) with constantly filtered and aerated water (at a height of 20 cm) at $20 \pm 1$°C. They were fed twice weekly with beef heart or commercial trout food (Provimi Agra, Zwolle, The Netherlands). Prior to induction of egg-laying, males and females were kept separate for at least 1 week at a temperature of 18°C. Fertilised eggs were obtained by subcutaneous injection of the adults with human chorionic gonadotropic hormone (hCG) (Organon, Oss, The Netherlands). Males were injected with 0.2 ml of 1500 IE hCG/ml subcutaneous (sc.). On the following day males were injected again with 0.2 ml of 1500 IE hCG/ml sc. and females received a single dose of 0.4 ml of 1500 IE hCG/ml sc. Thereafter pairs of adult animals were placed in aquaria at a temperature of 24°C overnight. On the next morning egg-laying had taken place. Thereafter adults were set apart and were not used for egg-laying for a period of 3 months.

Twelve adult (6/6) common frogs (*R. temporaria*) were collected on 30.3.1995 in Carinthia, Austria next to Maria Saal (46°38′30″N, 14°22′30″E). Males and females were separated and transported in boxes (10 l) on ice water covered with foam rubber immediately after collection to the Netherlands where they arrived 12 h later in good health. *R. temporaria* were kept at $17 \pm 1$°C throughout all experiments. After spawning adults were fed with commercially available living meal-worms ad libidum.

Tadpoles of both species were fed a diet consisting of 500 g of dried nettle powder, 5 g agar–agar, 5 g yeast powder and 5 g coffeecreamer. The powder was mixed in tempered water to get a viscous mixture, which was added once a day to the aquaria to achieve a dark green opaque colour. Water from all exposure experiments was charcoal-filtered prior to replacement.

*2.2. Chemicals*

3,3′,4,4′,5-PeCB (PCB126) (99% pure), respectively the technical mixtures Aroclor 1254 and Clophen A50 used for exposure of animals were obtained from Promochem (Wesel, Germany). 2-mercapto-1-methyl-imidazole (MMI) and the fixative NoTox were obtained from Janssen Chimica (Tilburg, The Netherlands) respectively from Earth Safe Industries ('s Hertogenbosch, The Netherlands). Human chorionic gonadotropic hormone (hCG) was obtained from Organon (Oss, The Netherlands). All other chemicals used throughout the experiments were of analytical grade and were obtained from Merck (Darmstadt, Germany). $T_4$ Amerlite kits were obtained from Amersham (Aylesbury, UK).

*2.3. Developmental studies on larvae after exposure of females to PCBs*

Clophen A50 was dissolved in cornoil in three concentrations: 0.01, 1.0, and 25 mg/ml. Adult *X. laevis* were anesthetized by keeping them for 5 min in icewater. 0.25 ml cornoil were applied into the stomach with a 1-ml syringe connected to a tube. Female *X. laevis* were dosed twice with 0.2 ml of cornoil 14 and 7 days prior to egg-laying. Control animals ($n = 5$) received cornoil only. Females weighed 80–100 g so that applied doses in the PCB treated animals of approximately 0.05 mg/kg ($n = 2$), 5 mg/kg ($n = 5$), and 125 mg/kg Clophen A50 ($n = 3$) were achieved. Seven days after the second dosage egg-laying was induced as described above. Collection and treatment of embryos was done according to the FETAX-protocol (ASTM, 1991). Further details on the FETAX procedure are given in subchapter 2.4. Embryos were kept for 96 h in Petri dishes according to the FETAX-protocol. Thereafter groups of 25 embryos (stage 46) without visible malformations were placed in aquaria (10 l) and their further development was registered until successful metamorphosis. *X. laevis* were scored for developmental stages according to Nieuwkoop and Faber (1975). In addition, from all females with enough embryos, 25 embryos were randomly selected, preserved in NoTox and scored for malformations under a stereomicroscope.

Six adult female *R. temporaria* were dosed once in the way described for *X. laevis* on the day of arrival. Three females received 0.2 ml cornoil only and three animals were dosed with 0.2 ml of cornoil with 25 mg Clophen A50/ml and kept for another 7 days at a temperature of 4 °C separated from the males. Males were kept at 17 ± 1°C during this period. After 7 days pairs of *R. temporaria* were placed in an aquarium at 17 ± 1°C to induce spawning which took place within 36 h. Thereafter eggs were placed in another aquarium. From every female 25 tadpoles (stage 25) were transferred into another aquarium and development until successful metamorphosis was followed as described for *X. laevis*. In addition 50 embryos of *R. temporaria* per clutch were sampled ad random (stage 25), preserved in NoTox and scored for malformations. Malformations of *R. temporaria* were determined using published descriptions of the normal staging as a pictorial guide (Gosner, 1960).

Eggs and embryos of females from both species were collected at five comparable stages of embryonic development for analysis of retinoids. These specimens were stored at −20°C prior to analysis.

*2.4. FETAX*

Eggs (stage 2 to stage 8) of *X. laevis* were prepared and scored according to ASTM (1991). Briefly eggs were collected and jelly layers of the eggs were removed by swirling the eggs for 2 min in FETAX buffer (625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4.2H_2O$ and 75 mg $SO_4.7H_2O$ per l distilled water) that contained L-cysteine–HCl (0.13 mol/l, adjusted to pH 8.0 with NaOH solution). The cysteine was removed by washing the eggs several times with FETAX-medium until the supernatant became clear. After scoring, groups of 25 embryos (< stage 13) without visible malformations were placed in 60-mm glass dishes with 10 ml FETAX-medium. All experiments were repeated in triplicate, using embryos from different sets of adults. The technical PCB-mixture Aroclor 1254 was used in concentrations ranging from 1.1 nM up to 1.2 mM and the non-ortho congener PCB 126 in a concentration range of 17.2 pM up to 15.3 μM all dissolved in dimethylsulfoxide (DMSO). Final DMSO concentrations were 0.5% in the PCB exposed and the vehicle control group. During the FETAX exposure period of 96 h, the dishes were kept at 24°C, the exposure media were replenished every day and dead animals were removed and counted. After 96 h animals had reached stage 46 and were fixed in ice-cold NoTox solution. They were scored for developmental anomalies and body length was measured using a stereomicroscope (ASTM, 1991).

*2.5. Prolonged-FETAX*

The prolonged-FETAX assay is based roughly on the FETAX procedure. Groups of 200 animals per concentration were exposed to PCB 126 (7.7 pM, 0.64 nM, 6.4 μM) for a 96 h period and were thereafter transferred into bigger aquaria. Animals were not further exposed to PCBs until the termination of the experiment after 80 days. Aquaria were checked every day and larvae found dead were fixed and scored for malformations (Bantle et al., 1991) under a stereomicroscope throughout the experiments. Animals, which had successfully

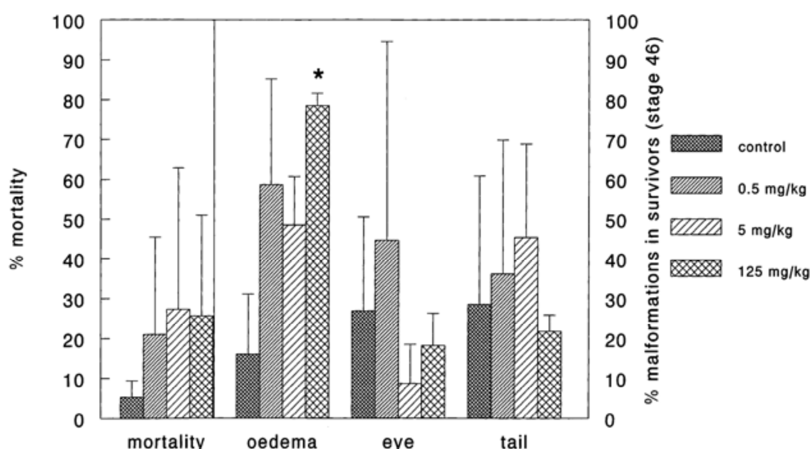


Fig. 1. Mortality and percentage of malformations in randomly selected embryos of *X. laevis* (stage 46) following female exposure to Clophen A50

undergone metamorphosis (stage 65/66) were anesthetised in ice-water and thereafter sacrificed by cervical transection, weighed and scored for malformations. Lower jaw tissues were removed and stored at −80°C prior to analysis of $T_4$.

### 2.6. Retinoid analyses

Retinoids in embryos were analysed according to Morse and Brouwer (1995) with some minor modifications. Embryos of the same stage (stage 10–12, $n = 10$; later stages, $n = 3$) were pooled in Eppendorf vials, homogenised in 50 mM Tris–HCl buffer, pH 7.5. Homogenates (50 µl) were vortexed with 50 µl methanol containing the internal standard (500 ng retinyl acetate/ml) and 0.1% 2,6-di-*tert*-butyl-4-methylphenol (BHT) (w/v) as an antioxidant. Samples were then extracted overnight at −20°C with 100 µl diisopropylether. The ether phase was removed and filtered over a 0.45-µm Millipore filter (Millipore, Etten Leur, The Netherlands) and dried under a gentle stream of nitrogen. The dried residues were dissolved in 50 µl methanol:ethyl acetate (3:1 with 0.1% BHT added). Extractions were carried out in duplicate.

Aliquots of 20 µl resuspended extracts were analysed with HPLC using a reversed phase silica RP C18 column (Pecosphere, 3 µm particle size, 3.3 cm length and 4.6 mm internal diameter, Perkin Elmer). A Spectra-Physics Analytical HPLC system was used consisting of a P2000 pump, UV-1000 UV–VIS detector, AS-3000 autosampler and a Merck–Hitachi D2500 Chromato-integrator. Retinoids were analysed by 85% methanol and 15% water with a flow rate of 1 ml/min for 1.5 min, followed by a gradient to 100% methanol for 2.5 min and subsequent elution of the retinyl esters at 100% methanol for 11 min. The column was then re-equilibrated at 85% methanol and 15% water for another 5 min (total run is 20 min). Extracts were analysed at two different wavelength settings. At first the UV–VIS detector was set at 380 nm for 4 min to detect all-*trans*-retinal and thereafter the later eluting substances such as all-*trans*-retinylpalmitate were analysed at 326 nm. In a second run the extracts were analysed at 326 nm to quantify all-*trans*-retinol. All-*trans*-retinol, all-*trans*-retinal, and all-*trans*-retinylpalmitate were quantified based on calibration curves. The putative 3,4-didehydro-retinylpalmitate was quantified using the standard for all-*trans*-retinylpalmitate and a correction factor of 1.64 (Stacher and Zonta, 1984). Calculations were corrected for the recovery rate.

Fig. 2. (a) *X. laevis* embryos (≤stage 22) of a control female approximately 24 h after fertilisation. (b) *X. laevis* embryos (≤stage 22) of a PCB dosed female (125 mg/kg Clophen A50). Note severe oedema and axial truncation. (c) *X. laevis* embryos (≤stage 46) of a control female approximately 96 h after fertilisation. (d) *X. laevis* embryos (≤stage 46) of a PCB dosed female (125 mg/kg Clophen A50). Note multiple severe oedema, axial shortening and retardation of gut coiling. (e) *X. laevis* embryo of a PCB dosed female (125 mg/kg Clophen A50). Note severe cardiac and abdominal oedema, reduction in eye size, small rounded head, incomplete gut coiling and complex axial malformation. (f) *X. laevis* embryo of a PCB dosed female (125 mg/kg Clophen A50). Note severe cardiac and abdominal oedema, small rounded head, incomplete gut coiling and complex axial malformation including a doubling of the tail. (f) *R. temporaria* embryos (≤stage 20) of a PCB dosed female (125 mg/kg Clophen A50). Note complex axial malformations with a wavy notochord. (h) *R. temporaria* embryos of a PCB dosed female (125 mg/kg Clophen A50). The embryo on the bottom is the control embryo. Note complex axial malformations with a wavy notochord of the next two embryos. On top four embryos with a loss of the anterior posterior axis.

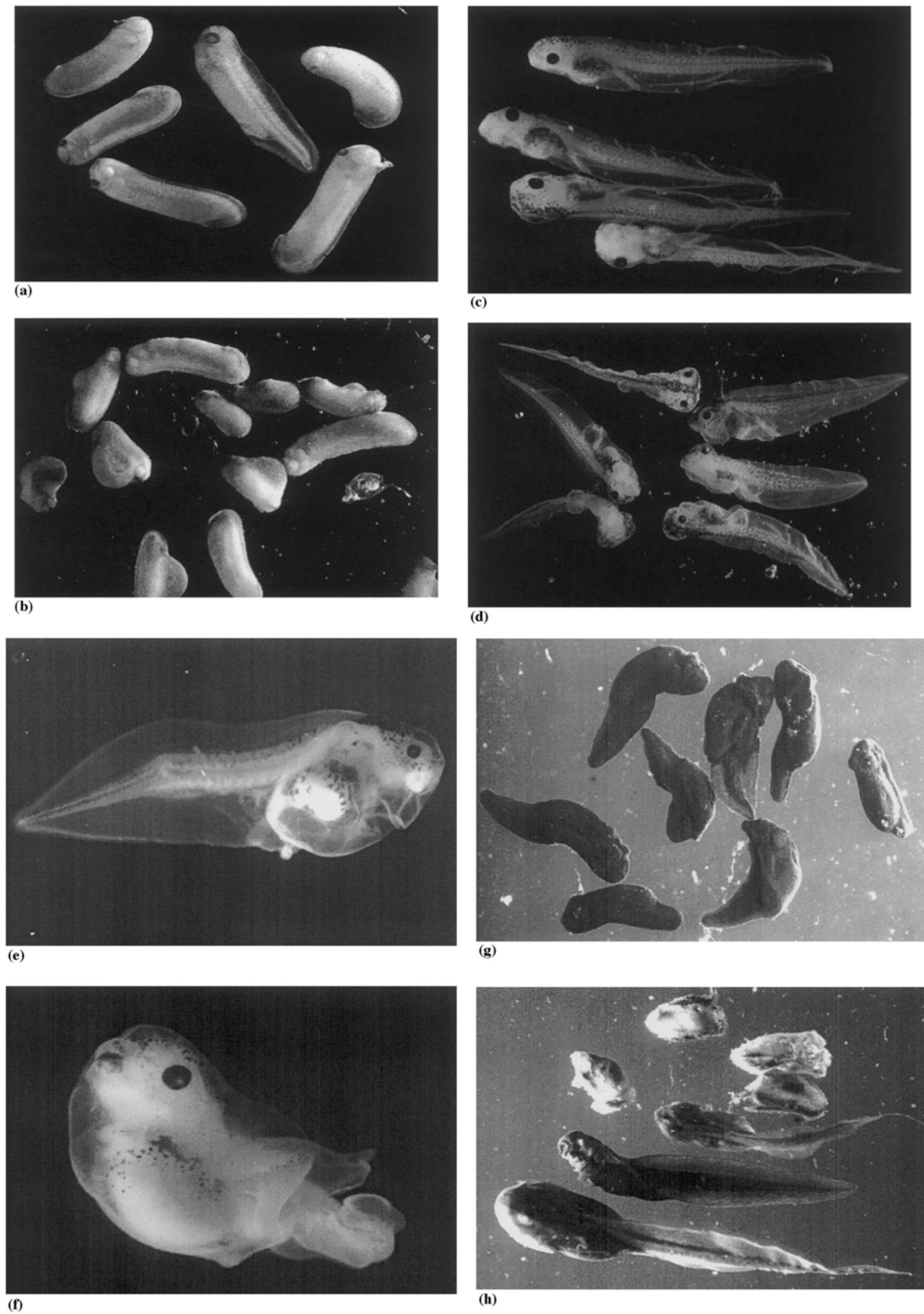


Fig. 2.

*2.7. Thyroid hormone assays*

Total thyroxin ($T_4$) levels were determined following the method of Bray and Sicard (1982) with a few modifications. The lower jaws of tadpoles containing the diffuse thyroid follicles were freeze dried (CMC1 Freeze drier Christ Alpha RVS.X.IKS, Leerdam, The Netherlands) overnight. Freeze dried lower jaws were manually homogenised in an Eppendorf vial. After adding 100 µl methanol and 10 min vortexing, the vials were stored overnight at 4°C. The next morning samples were vortexed for 10 min, centrifuged with 6000 rpm and the supernatant was transferred into another vial. After adding 100 µl ammonialcale ethanol to the pellet the extraction procedure was repeated. The combined supernatants were dried under a stream of nitrogen and dissolved in 20 µl $T_4$-free serum. In the extracts $T_4$ was determined by chemiluminescence, using the Amerlite system (Amersham, UK) and the protocol of the supplier with slight modifications. The $T_4$ assay reagent was diluted five times with demineralised water and the $T_4$ standard curve ranged from 0 to 30 nM $T_4$/l. Thyroid hormone levels were calculated from the luminescence data with the Securia computer program of Amersham.

*2.8. Statistical analyses*

All data are reported as means ± SEM. Differences between means were tested with one-way ANOVA respectively Student's *t*-test where appropriate. The acceptance level was set at $P < 0.05$. Data that did not pass the Levene's test for normality were analysed for differences to control groups either using Mann–Whitney U-test or Kruskal–Wallis H-test. All statistical analysis were performed using SPSS/PC+, version 6.0 (SPSS Inc., Chicago, IL, USA).

## 3. Results

*3.1. Developmental studies on tadpoles from PCB exposed female amphibians*

*3.1.1. X. laevis*

Mortality during the first 96-h following egg-laying in randomly selected embryos from PCB-dosed females was increased (Fig. 1) although differences were not significant (Kruskal–Wallis H-test, $P > 0.05$). Severe oedema was observed already 24 h after fertilisation in randomly selected embryos (≤ stage 22) from PCB dosed females (Fig. 2a and b) and the overall incidence of oedema was increased in embryos at the end of the 96-h observation period irrespective of the female PCB dose (Fig. 1). In the offspring from the high dosed females (125 mg/kg Clophen A50), the rate of embryos with oedema was significantly increased (Kruskal–Wallis H-test, $P < 0.05$) when compared with the control group (Fig. 2c and d). Several embryos from the high dosed females were severely misformed (Fig. 2e and f) and this type of malformation was never observed in control groups.

Mortality in *X. laevis* tadpoles of the PCB-dosed females, which have been selected as embryos according to the FETAX-protocol, was not increased (Fig. 3) until successful metamorphosis in any of the dosed groups compared with the control group (Kruskal–Wallis H-test, $P > 0.05$). Incidence of malformations in tadpoles, which died throughout the larval period was highest in offspring of females which received the highest Clophen A50 dose (125 mg/kg), where 73.3% of the embryos showed at least one type of malformation. In the other two PCB exposed groups the overall percentage of malformations was 72.7% (5 mg/kg) and 52.9% (0.05 mg/kg), which is higher than in the control group (50%). The pattern of malformations was similar

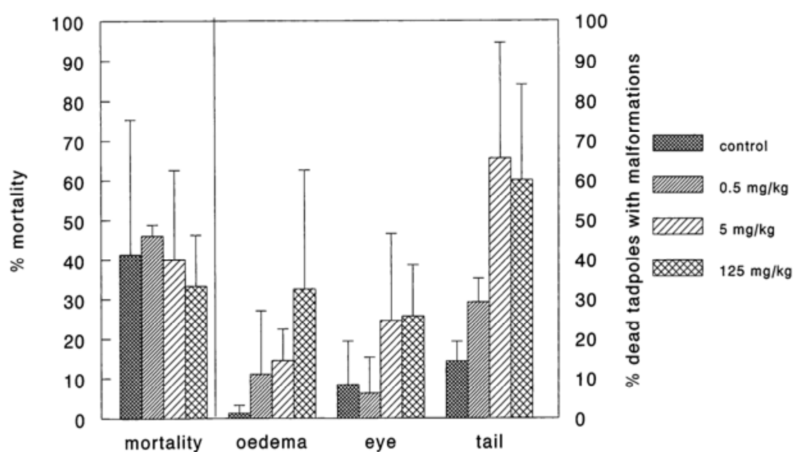


Fig. 3. Mortality and percentage of malformations in dead tadpoles during the larval period (stage 46–66) in embryos of *X. laevis* following female exposure to Clophen A50.

Table 1
Body weight and larval period until successful metamorphosis of *X. laevis* from PCB exposed females[a]

| | Froglets *n* | Body weight (mg) | Larval period (days) |
|---|---|---|---|
| Control | 34 | $280 \pm 23$ | $74 \pm 2$ |
| 0.05 mg/kg | 30 | $198 \pm 10^{b}$ | $79 \pm 4$ |
| 5 mg/kg | 20 | $211 \pm 27^{b}$ | $73 \pm 4$ |
| 125 mg/kg | 20 | $227 \pm 21^{b}$ | $76 \pm 2$ |

[a] Statistical evaluation (body weight, larval period) was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Scheffé's test that controlled Type I errors,
[b] $P < 0.05$.

in all groups with tail deformities being the most prominent malformation (Fig. 3).

No significant differences were observed between any of the groups for the period until successful metamorphosis (one-way ANOVA, $P > 0.05$) (Table 1).

At the end of successful metamorphosis tadpoles body weight from all Clophen A50 treated females was significant lower than in the control group (one-way ANOVA, $P < 0.05$), however this was not dose-related (Table 1).

Retinoids (vitamin A) were analysed in *X. laevis* whole embryo homogenates by reversed phase (C-18) HPLC method (Fig. 4a and b). All-*trans*-retinol, all-

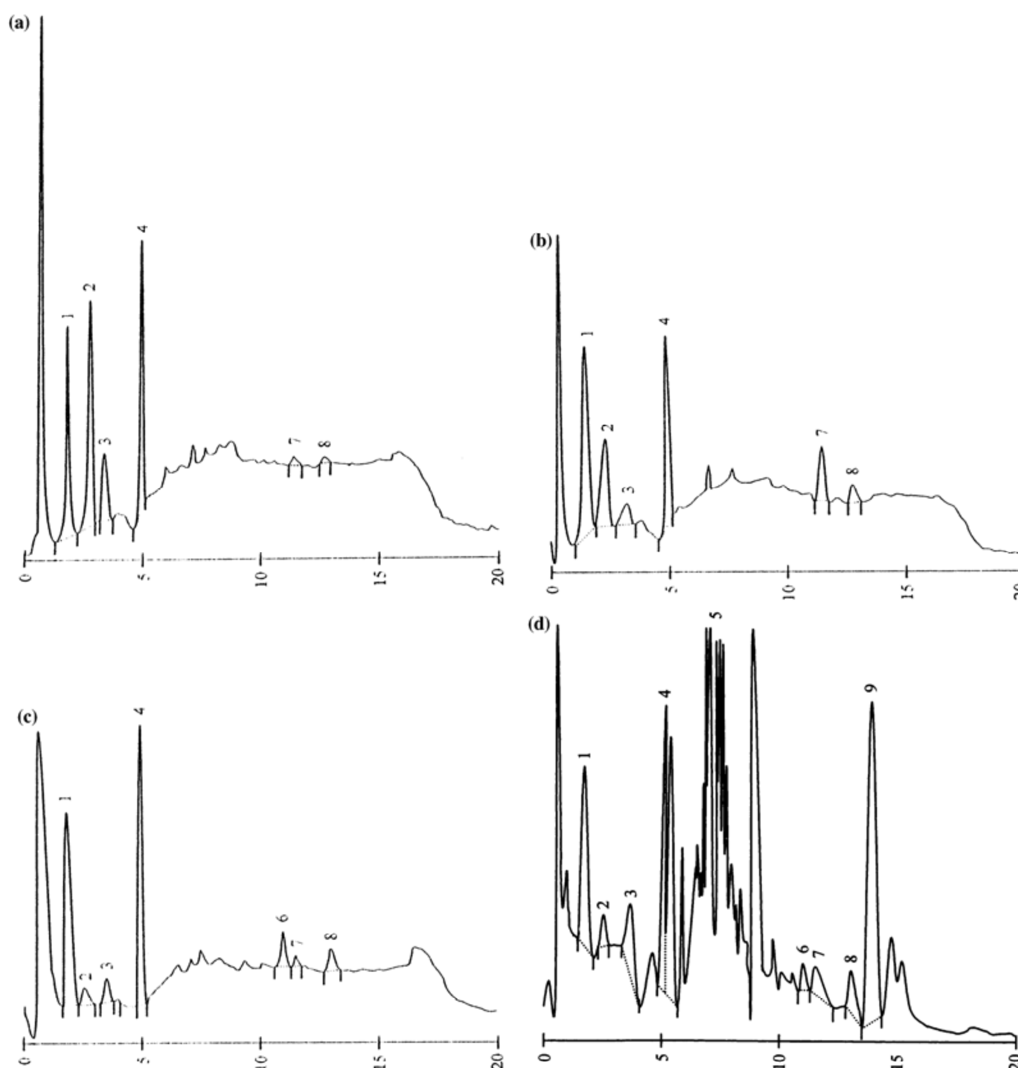


Fig. 4. Typical HPLC chromatograms for retinoids in unexposed *X. laevis* embryos (a, c) and *R. temporaria* embryos (b, d). The upper panel shows retinoid profiles obtained by reversed phase silica C18 HPLC for stage 10–12 (a, b) and the lower panels for stages 46 (*X. laevis*) respectively stage 25 (*R. temporaria*). HPLC chromatographic conditions are given in subchapter 2.6. 1, BHT; 2, 3,4-all-*trans*-didehydro-retinal; 3, all-*trans*-retinal; 4, retinylacetate (internal standard); 5, group of unknown peaks; 6, possibly an ester with a shorter fatty acid than palmitate; 7, 3,4-didehydroretinylpalmitate; 8, all-*trans*-retinylpalmitate; 9, all-*trans*-retinylstearate.

Table 2
Retinoid concentrations in *X. laevis* embryos (pM/animal) after exposure of females to Clophen A50[a]

| Stage | Dosage (mg/kg) | all-*trans*-retinal | all-*trans*-retinol | all-*trans*-retinyl-palmitate | 3,4-didehydro-retinylpalmitate | Ratio all-*trans*-retinol/all-*trans*-retinylpalmitate (ratio · 100)[c] |
|---|---|---|---|---|---|---|
| 10–12 | Control | 5.04 ± 0.73 | NA | 0.27 ± 0.11 | 0.31 ± 0.06 | – |
| | 5 | 3.58 ± 0.53 | NA | 0.36 ± 0.11 | 0.34 ± 0.07 | – |
| | 125 | 5.39 ± 0.73 | NA | 0.15 ± 0.03 | 0.29 ± 0.03 | – |
| 29/30 | Control | 3.44 ± 0.94 | NA | 0.21 ± 0.03 | 0.64 ± 0.09 | – |
| | 5 | 3.74 ± 1.41 | NA | 0.23 ± 0.02 | 0.57 ± 0.10 | – |
| | 125 | 5.93 ± 1.33 | NA | 0.23 ± 0.02 | 0.57 ± 0.06 | – |
| 39 | Control | 4.00 ± 0.72 | 1.04 ± 0.43 | 0.85 ± 0.23 | 1.74 ± 0.66 | 81 ± 98 |
| | 5 | 4.55 ± 0.72 | 1.83 ± 0.53 | 1.10 ± 0.25 | 1.36 ± 0.62 | 106 ± 97 |
| | 125 | 7.29 ± 0.87[b] | 0.83 ± 0.45 | 0.73 ± 0.23 | 1.60 ± 0.29 | 83 ± 133 |
| 43 | Control | 2.56 ± 0.36 | 0.15 ± 0.05 | 0.59 ± 0.07 | 3.23 ± 0.41 | 28 ± 27 |
| | 5 | 3.22 ± 0.44 | 0.44 ± 0.13 | 0.60 ± 0.05 | 2.80 ± 0.35 | 66 ± 54 |
| | 125 | 3.26 ± 0.38 | 0.58 ± 0.21 | 0.62 ± 0.07 | 2.74 ± 0.26 | 73 ± 72 |
| 46 | Control | 1.26 ± 0.39 | 1.01 ± 0.13 | 0.74 ± 0.05 | 4.32 ± 0.25 | 134 ± 28 |
| | 5 | 1.58 ± 0.20 | 0.97 ± 0.13 | 0.68 ± 0.08 | 3.61 ± 0.71 | 144 ± 36 |
| | 125 | 1.50 ± 0.34 | 0.85 ± 0.25 | 0.75 ± 0.12 | 3.80 ± 0.44 | 106 ± 42 |

[a] NA, not analysed; statistical evaluation was made by Kruskal–Wallis H-test,
[b] $P<0.05$;
[c] Mean ± SD.

*trans*-retinal, and all-*trans*-retinylpalmitate were detected in the homogenates of embryos from all stages analysed. In the first stage analysed (stage 10–12) all-*trans*-retinal, all-*trans*-retinylpalmitate, the putative 3,4-didehydro-retinal, and presumably the corresponding ester 3,4-didehydro-retinylpalmitate were present.

Retinoid analysis has not been carried out in embryos of the two females from the low PCB dosed groups of *X. laevis* as embryo numbers were too small. Levels of retinoids in total embryo homogenates were not altered significantly compared to cornoil-treated control animals with the exception of stage 39 embryos of the high dosed females (125 mg/kg Clophen A50) where increased levels of all-*trans*-retinal (Kruskal–Wallis H-test, $P > 0.05$) were found (Table 2). The levels of all-*trans*-retinal in embryos of the high dosed females at stage 10–12, 29/30, and 43 were also higher than in controls although not significant (Kruskal–Wallis H-test, $P > 0.05$). No significant differences for the ratio all-*trans*-retinol:all-*trans*-retinylpalmitate were found between any of the groups (Kruskal–Wallis H-test, $P > 0.05$).

### 3.1.2. *R. temporaria*

*R. temporaria* females were either exposed to the highest PCB concentration (125 mg/kg Clophen A50) used in the experiment with *X. laevis* or served as cornoil treated control animals. Randomly selected larvae (stage 25) from PCB exposed *R. temporaria* females (125 mg/kg Clophen A50) showed severe truncation with loss of the anterior–posterior axis or tail malformations (Fig. 2g and h, Fig. 5) although differences were not significant (Mann–Whitney U-test, $P > 0.05$). In the control groups no severely malformed larvae were found at all. Offspring from PCB exposed females showed a higher incidence of malformations (33.8%) than larvae from cornoil-treated control females (4.9%) (Fig. 5). The overall incidence of oedema was less prominent than in *X. laevis*.

No mortality was observed in maternally PCB exposed *R. temporaria* tadpoles and in the tadpoles from one cornoil-treated control female. Tadpoles from the second control female showed a steadily increasing mortality throughout development. No malformations in any of the groups were observed upon macroscopic inspection in dead tadpoles respectively in the froglets at the end of successful metamorphosis.

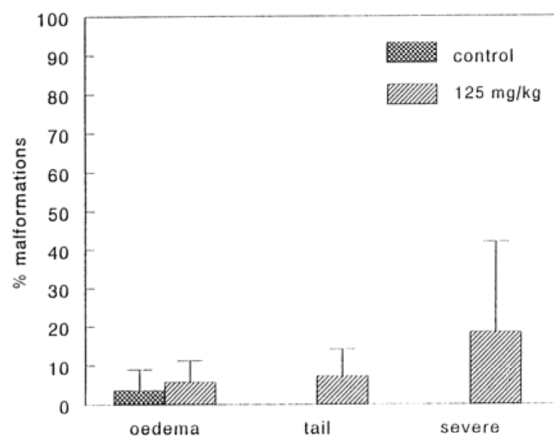


Fig. 5. Percentage of malformations in randomly selected embryos of *R. temporaria* (stage 25) following female exposure to Clophen A50.

Table 3
Body weight and larval period until successful metamorphosis of metamorphosed *R. temporaria* froglets from PCB exposed females

| | froglets *n* | Body weight (mg) | Larval period (days) |
|---|---|---|---|
| Control | 31 | $374 \pm 21$ | $45 \pm 1$ |
| 125 mg/kg | 82 | $311 \pm 6$[a] | $46 \pm 1$ |

[a] Student's *t*-test, $P<0.05$

Body weights of metamorphosed froglets from PCB treated females however were significantly lower (Mann-Whitney U-Test, $P < 0.05$) than froglets from control females (Table 3). The time period of development until successful metamorphosis did not differ significantly between the offspring from the cornoil-treated control groups and PCB dosed females (Mann–Whitney U-test, $P > 0.05$) (Table 3).

All-*trans*-retinol, all-*trans*-retinal, and all-*trans*-retinylpalmitate were detected in homogenates of *R. temporaria* embryos although in different concentrations when compared with *X. laevis* (Fig. 4c and d). In addition small differences in the presence of unidentified peaks between the two species are obvious in the first stage analysed (Fig. 4a and c). Peak 6 in Fig. 4c represents possibly an ester with a shorter fatty acid than palmitate. At stage 46 in *X. laevis* respectively the corresponding stage 25 in *R. temporaria* differences between the two species with respect to their retinoid profile have become more obvious (Fig. 4b and d). The group of peaks (peak 5 in Fig. 4d) is yet of unknown origin although present in all homogenates analysed. Peak 9 in Fig. 4d of *R. temporaria* probably is an ester of all-*trans*-retinol or 3,4-didehydro-retinol and a fatty acid longer than palmitate, presumably stearate.

Total body concentrations of all-*trans*-retinol in embryos from PCB dosed females (Fig. 6a) were significantly higher at stage 23 and 25 than in control animals (Mann–Whitney U-test, $P < 0.05$). Contrary to *X. laevis* embryos, all-*trans*-retinylpalmitate levels were significantly higher in offspring of Clophen A50 treated females (Fig. 6b) of *R. temporaria* from stage 10 up to stage 23 (Mann–Whitney U-test, $P < 0.05$). Concentrations of all-*trans*-retinal were also higher in offspring of PCB dosed females than in control animals although not significant (Mann–Whitney U-test, $P < 0.05$) (Table 4). In early embryos (stage 10–12 and stage 18) the ratio all-*trans*-retinol:all-*trans*-retinylpalmitate was significantly lowered in offspring of the PCB dosed females (Mann–Whitney U-test, $P < 0.05$).

### 3.2. FETAX assay

Neither Aroclor 1254, nor the single congener PCB 126 had an effect on growth and development in the FETAX assay when *X. laevis* eggs were exposed to a concentration range from 1.1 nM to 1.2 mM for Aroclor 1254 respectively 17.2 pM to 15.3 μM for PCB 126. It was not possible to determine an LC50 value for malformations and no teratogenicity index (TI) or a minimal concentration to inhibit growth (MCIG) could be calculated. The only observed effect was depigmentation of animals exposed to Aroclor 1254 in the μM range.

### 3.3. Prolonged-FETAX assay

5 days after ending the exposure period of *X. laevis* embryos, mortality increased sharply in the group exposed to 6.4 μM PCB 126 during the 96-h FETAX exposure period. Within the first 10 days after termination of exposure 58 animals or 29% had died and 95 (47.5%) died over the experimental period of 80 days. 3 weeks after the end of exposure tadpoles started to become pale and showed swimming disorders in the middle dosed group (0.64 nM PCB 126). A steady increase in mortality resulted in 43 dead animals

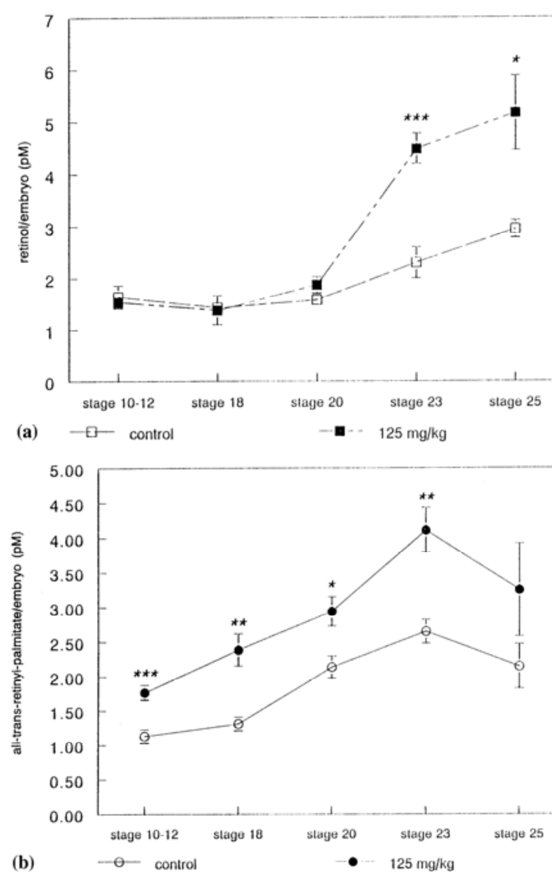


Fig. 6. Concentrations of all-*trans*-retinol (a) and all-*trans*-retinylpalmitate (b) in *R. temporaria* embryos of different stages.

Table 4
Retinoid concentrations in *R. temporaria* embryos (pM/animal) after female exposure to Clophen A50

| Stage | Dosage (mg/kg) | all-*trans*-retinal | all-*trans*-retinol | all-*trans*-retinyl-palmitate | 3,4-didehydro-retinylpalmitate | Ratio all-*trans*-retinol/all-*trans*-retinylpalmitate (ratio · 100)[d] |
|---|---|---|---|---|---|---|
| 10–12 | Control | 2.32 ± 0.08 | 1.64 ± 0.22 | 1.13 ± 0.10 | 0.28 ± 0.06 | 146 ± 45 |
| | 125 | 2.90 ± 0.69 | 1.54 ± 0.12 | 1.77 ± 0.11[c] | 0.26 ± 0.03 | 87 ± 19[b] |
| 18 | Control | 0.60 ± 0.32 | 1.43 ± 0.12 | 1.31 ± 0.10 | 0.93 ± 0.35 | 108 ± 37 |
| | 125 | 2.48 ± 1.17 | 1.38 ± 0.28 | 2.39 ± 0.23[b] | 0.65 ± 0.08 | 57 ± 29[a] |
| 20 | Control | 0.72 ± 0.30 | 1.58 ± 0.08 | 2.14 ± 0.16 | 1.03 ± 0.04 | 76 ± 19 |
| | 125 | 0.44 ± 0.19 | 1.87 ± 0.16 | 2.94 ± 0.21[a] | 1.04 ± 0.07 | 65 ± 14 |
| 23 | Control | 0.60 ± 0.28 | 2.30 ± 0.30 | 2.65 ± 0.17 | 2.52 ± 0.24 | 89 ± 33 |
| | 125 | 0.81 ± 0.44 | 4.49 ± 0.29[b] | 4.11 ± 0.32[b] | 3.03 ± 0.19 | 116 ± 37 |
| 25 | Control | 0.51 ± 0.26 | 2.95 ± 0.17 | 2.15 ± 0.32 | 1.74 ± 0.13 | 161 ± 63 |
| | 125 | 0.98 ± 0.42 | 5.17 ± 0.71[a] | 3.25 ± 0.67 | 1.95 ± 0.28 | 166 ± 58 |

[a] Mann–Whitney U-test, $P<0.05$;
[b] $P<0.001$;
[c] $P<0.01$
[d] mean ± SD

(21.5%) in this exposure group over the whole experimental period. In the low dose group exposed to 7.7 pM PCB 126 a total of 21 animals died showing also symptoms of swimming disorders. In addition to these dose dependent effects acute mortality without preceding symptoms was observed in the untreated control group ($n=95$) and later in the DMSO treated vehicle control group ($n=91$). The mortality was observed after the aquaria have been cleaned by staff members using Latex gloves starting the cleaning procedure in the aquarium of the respective group.

Only 7.3 and 8.4% of the dead animals in the control and DMSO treated group showed malformations. However a dose-related increase in the number of malformations was found in the groups exposed to PCB 126 (Fig. 7). This effect was most prominent in the group exposed to 6.4 μM PCB 126 with 93.2% of malformed animals and declined down to 45% in the group exposed to 7.7 pM PCB 126. Oedema, misformed eyes and tail, and lack of gut coiling were the most prominent observed malformations (Fig. 8). Eye malformations included reduction in size, failure of the chorioid fissure to close, rupture of the optic cup and irregular depigmentation.

Length of larval period until successful metamorphosis ranged from 60 days in the group exposed to 7.7 pM PCB 126 to 68 days in the group exposed to 0.64 nM PCB 126 (Table 5). The differences between the groups were not significant (one-way ANOVA, $P>0.05$).

Mean body weight in metamorphosed animals ranged from $180 \pm 8$ to $225 \pm 20$ mg (Table 5). No statistical significant differences between the groups were found (one-way ANOVA, $P>0.05$).

A dose-related decrease of $T_4$ concentrations in the lower jaws of the froglets at stage 65/66 ranging from 35% in the high dosed group (6.4 μM PCB 126) to 24% in the low dosed group (7.7 pM PCB 126) was observed although not significant (Kruskal–Wallis H-test, $P>0.05$) (Table 5).

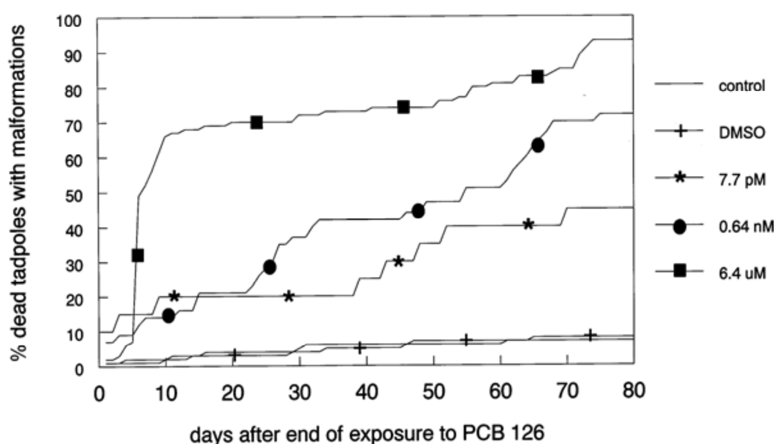


Fig. 7. Percentage of dead tadpoles of *X. laevis* with malformations after 96-h exposure to PCB 126 from stage 13 to stage 46 (prolonged FETAX).

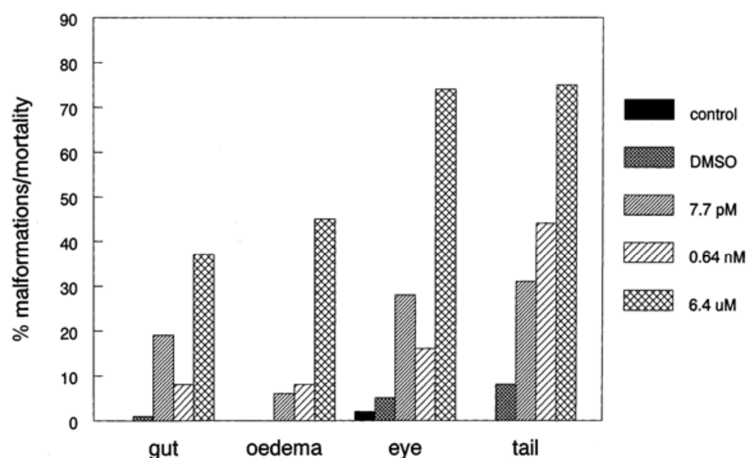


Fig. 8. Percentage of malformations in dead tadpoles of *X. laevis* after 96-h exposure to PCB 126 from stage 13 to stage 46 (prolonged FETAX).

Table 5
Body weight, larval period until successful metamorphosis, and $T_4$ levels in thyroid tissue of metamorphosed *X. laevis* after exposure to PCB 126 as embryos (prolonged FETAX)[a]

| | *n* | Larval period (days) | Body weight (mg) | *n* | $T_4$ (pg/lower jaw; mean ± SD)[b] |
|---|---|---|---|---|---|
| Control | 32 | 61 ± 2 | 196 ± 11 | 3 | 36 ± 12 |
| DMSO | 14 | 61 ± 2 | 180 ± 8 | 3 | 37 ± 12 |
| 7.7 pM | 29 | 60 ± 2 | 188 ± 14 | 3 | 28 ± 8 |
| 0.64 nM | 15 | 68 ± 3 | 225 ± 20 | 3 | 26 ± 16 |
| 6.4 µM | 10 | 61 ± 2 | 204 ± 19 | 3 | 24 ± 14 |

[a] Statistical evaluation (body weight) was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Scheffé's test that controlled Type I experimentwise error,
[b] Statistical evaluation for $T_4$ concentrations were made by Kruskal–Wallis H-test.

## 4. Discussion

PCBs are known to induce a plethora of effects in adults as well as in juvenile and developmental stages of vertebrate species. One hypothesis for the mechanism of induction of developmental effects is through disruption of hormonal systems such as thyroid hormone and retinoids. In fact disturbance of the retinoid pattern and thyroid hormone homeostasis has been observed as a result of PCB exposure in other vertebrate species. Since both retinoids and thyroid hormone are of vital importance for normal amphibian development and long-term population recruitment, it may be possible that PCBs affects these processes through an endocrine disrupting mechanism of actions. The current study was undertaken to evaluate possible effects of PCBs on the development of tadpoles. The focus of the study was on the influence of early embryonic exposure to PCBs on metamorphosis through maternal exposure just before egg laying, or by directly exposing the fertilised eggs. Thyroid hormone, retinoids, period of metamorphic transformation, body weight, mortality and malformations were analysed in embryos after pre- and early-life time exposure to PCBs (Table 6).

Table 6
Effects of PCBs on *X. laevis*/*R. temporaria* offspring as a result of different exposure routes (NE, no effect; ND, not determined; NAP, not applicable)

| Effect | Route of exposure | | |
|---|---|---|---|
| | Female | Egg (FETAX) | p-FETAX |
| Mortality | Up[a]/NE | NE/– | Up[b]/ND |
| Malformation | Up[b]/Up[c] | NE[d]/– | Up[b]/ND |
| Body weight | Down[b]/Down[c] | NE[e]/– | NE/ND |
| Larval period | NE/NE | NAP/– | Up[b]/ND |
| $T_4$ | ND/ND | ND/– | Down[a]/ND |
| Retinoids | Up[b]/Up[c] | ND/– | ND/ND |

[a] *X. laevis* trends
[b] *X. laevis* significant effects;
[c] *R. temporaria* significant effects;
[d] Depigmentation has been observed;
[e] Length.

### *4.1. Effect on mortality*

Randomly selected *X. laevis* embryos from Clophen A50 exposed females exhibited increased mortality (Table 6) whereas *X. laevis* tadpoles, which were carefully selected to be free of visible malformations according to the FETAX-protocol and *R. temporaria* tadpoles showed no increase in mortality. The increased mortality in maternally exposed *X. laevis* offspring is remarkable as the PCB concentration (sum of congeners 28, 52, 101, 118, 138, 153, and 180) of 0.8 mg/kg fat weight in livers of the lowest dosed group (data not given), is in the same order of magnitude than field data for amphibians (Leonards, 1997).

Time delayed increase in the incidence of mortality was observed in the PCB dosed groups in the prolonged FETAX assay (Table 6). This delayed onset of mortality even weeks after termination of exposure to toxic compounds, is in accordance with data of McKinney et al. (1985), although *X. laevis* tadpoles used in their experiments have been exposed for the much longer period (30 days) to 100 ng/l of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In carp (*Cyprinus carpio*) in the swim-up stage after resorption of the yolksac an oedematous syndrome has become apparent (Stouthart et al., 1998). PCB 126, which is likely retained in the triglyceride fraction of yolk, may therefore not be readily available for toxic action. The 96-h exposure period in the conventional FETAX assay seems to be too short to detect subtle effects of substances with a low acute toxicity, whereas in the newly developed prolonged FETAX assay a dose-dependent mortality occurred beginning 5 days after PCB exposure had stopped.

Shortly after the two incidents with increased mortality connected to the cleaning procedure in the control and the DMSO group, such acute mortality of *X. laevis* larvae resulting from LATEX gloves was described by Sobotka and Rahwan (1994).

### *4.2. Effect on body weight, duration of larval period and thyroid hormone levels*

Body weight was significantly reduced in maternally exposed tadpoles of both species whereas body weight was not altered in *X. laevis* froglets exposed in the prolonged FETAX assay (Table 6). There is some evidence that interference of PCBs with *X. laevis* tadpole thyroid hormone homeostasis occurred under our experimental conditions as $T_4$ levels in tadpoles were lowered although not significant even weeks after PCBs exposure has been terminated in the prolonged FETAX assay. At the time of thyroid hormone analysis PCB levels in these animals were lower than the detection limit (data not given). Alterations in $T_4$ levels are expected to affect the thyroid hormone dependent amphibian metamorphosis (Bray and Sicard, 1982), which will consecutively be followed by changes in body weight of successfully metamorphosed froglets.

Metamorphosis occurred at a larger body size, which can increase survival of terrestrial juveniles and size at first reproduction and therefore fecundity on an individual but also on a population scale (Berven and Gill, 1983). Semlitsch et al. (1988) were able to show that amphibian individual lifetime fitness was negatively affected by the tadpoles environment. Several publications reported that smaller tadpole body size is correlated with alterations of swimming speed, which resulted in increased predation (Feder, 1983; Richards and Bull, 1990).

### *4.3. Malformations and retinoid concentrations*

In *X. laevis* a dose-dependent increase in the incidence of eye and tail deformities in the prolonged FETAX assay was found (Table 6). These results are similar to what was found after TCDD exposure during early embryo development in amphibians and fish (Birge et al., 1978; Elonen et al., 1998).

The ratio all-*trans*-retinol:all-*trans*-retinylpalmitate, a measure for the mobilisation rate of all-*trans*-retinol from hepatic retinoid stores, is decreased in *R. temporaria* embryos from PCB dosed females, which is an indication for reduced metabolisation of hepatic retinoid esters in adult females, most probably as a result of increased hepatic all-*trans*-retinol metabolism. Any alteration in the subtle balance of metabolisation and transportation of active and storage forms of retinoids in females will lead consecutively to alterations of retinoid concentrations in eggs, thereby disturbing early embryo development. Similar stunted animals with oedema were found after early embryonic exposure to retinoids respectively retinoid-X-receptor selective ligands in *X. laevis* (Creech-Kraft and Juchau, 1995; Durston et al., 1989; Pijnappel et al., 1993).

The time shift in retinoid concentrations at different stages in *X. laevis* and *R. temporaria* gives further evidence for the ability of embryos of both species to metabolise retinoids as this was shown for *X. laevis* by Creech-Kraft et al. (1995) and for *R. catesbeiana* by Tsin et al. (1985). In mammals, retinoid disturbing effects of PCBs are reported to result either from interference of hydroxy-metabolites with the plasma transport of all-*trans*-retinol by transthyretin (Brouwer and van den Berg, 1986), or from modulation of retinoid metabolising enzymes (Zile, 1992). In amphibians, these two mechanisms have not been studied in detail, although there is some evidence that in adult *Rana catesbeiana* these mechanism are present (Shidoji and Muto, 1977).

### 4.4. *Abberations*

Development of oedema seems to be a common reaction to toxicants in amphibians as oedema was also found in *R. pipiens* and *Bufo americanus* within 24 h after exposure to alachlor and atrazine had started (Howe et al., 1998). Mima et al. (1992) exposed *X. laevis* to concentrations of 100, 200, and 400 ng TCDD/ml whereafter 77% of the tadpoles produced oedema at day 5, and 76% of these animals died later in their development. PCB 126 induced an increase of adrenocorticotropic hormone (ACTH) and cortisol in carp (*C. carpio*), which coincided with development of oedema, thus giving evidence for possible effects of PCBs on the water balance (Stouthart et al., 1998).

In addition to the above mentioned malformations and abberations, a dose-dependent depigmentation in embryos exposed for 96-h to Aroclor 1254 (FETAX) has been observed. In leopard frog (*R. pipiens*) and green frog (*R. clamitans*) tadpoles similar effects of depigmentation were observed following 24-h waterborn exposure to $\geq 0.3$ μg TCDD/L (Jung and Walker, 1997).

## 5. Conclusions

The results presented in this paper show, that effects of PCB exposure in amphibians such as mortality, number and pattern of malformations, or body weight at the end of successful metamorphosis of tadpoles, depends on the route, the point of time of exposure during the complex life cycle of amphibians, and the length of the observation period. In our experiments we found similarities in the effects of PCB exposure on *X. laevis* and *R. temporaria* exposed via the same route. The combination of the effects of pre- and early-lifetime exposure in conjunction with the observed effects of oral exposure to PCBs (Gutleb et al., 1999) on individual lifecycles respectively on the population level cannot be judged with present knowledge of the field situation in respect to population structure and recruitment.

Furthermore we conclude that presently used early-life time test systems such as the FETAX assay may underestimate toxic effects of compounds with long term response such as PCBs on amphibians.

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# 3

# The prolonged-FETAX detects effects of exposure to environmentally relevant concentrations of POPs that are not detected in the FETAX

[*submitted*]

*Xenopus laevis* embryos (NF stage 24 - 35)

# CHAPTER 3

## The prolonged-FETAX detects effects of exposure to environmentally relevant concentrations of POPs that are not detected in the FETAX

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### Abstract

The prolonged-FETAX (prolonged-Frog Embryo Teratogenic Assay - Xenopus), in which the tadpoles are allowed to develop until metamorphosis after initial 4 day-exposure, was applied to test delayed long-term effects of early-life time exposure of *Xenopus laevis* embryos to PCB 77 (3,4,3′,4′-tetrachlorobiphenyl) and sediment extracts. The results were compared to those obtained with the standard FETAX.
In the prolonged-FETAX significant changes in the percentage of animals that passed metamorphosis were found with decreased percentages of metamorphosed animals after 115 days in the groups having been exposed to 300 or 30,000 nM PCB 77. The water volume per animal, however, must be kept constant to overcome the influence of mortality and metamorphosis on the density dependent development of the remaining tadpoles. Significant increased percentages of tadpoles halted in thyroid hormone dependent early metamorphic stages <56 (3 nM PCB 77) and metamorphic stages 56-60 tadpoles (300 nM PCB 77 and 30,000 nM PCB 77) were found, mirrored by an increased number of development dependent awarded penalty points in the PCB 77-exposed groups. Apolar sediment extracts from two contaminated Dutch sites significantly decreased the percentage of animals that passed metamorphosis, with dose-dependent increase of penalty points. All these results strongly point to the presence of thyroid hormone disrupting compounds in the aquatic environment. Exposure to PCB 77 and apolar sediment extracts did not result in any effects in the classical FETAX. However altogether, the results of the present study indicate that the prolonged-FETAX is an important additional tool to assess long-term effects of early life stage exposure to low and environmentally relevant concentrations of compounds and their mixtures that affect thyroid hormone dependent physiology.

Keywords – prolonged-FETAX, amphibians, development, bioassay

## INTRODUCTION

The Frog Embryo Teratogenesis Assay-Xenopus (FETAX) test, a short-term early-life time test has been shown to be a powerful bioassay for the identification of compounds that exhibit embryotoxic potency (Fort and Bantle, 1990; Fort et al., 2000; Fort and Paul, 2002; Mann and Bidwell, 2000; Luo et al., 1993; Bernardini et al., 1996; Herkovits et al., 1997; Bantle et al., 1994a,b; Dumont et al., 1983). Nevertheless FETAX was shown to underestimate long-term effects of persistent compounds with low acute embryotoxicity such as polychlorinated biphenyls (PCBs) that were detected by the prolonged-FETAX (Gutleb et al., 1999). No effect on mortality, rate of malformations, growth, or development of *Xenopus laevis* embryos were observed with the FETAX scored on day 4 at Nieuwkoop and Faber (1975) NF stage 46 in a concentration range up to 15.3 μM PCB 126 (Gutleb et al., 1999). When animals were not sacrificed and scored on day 4 but rather placed over into aquaria and were kept unexposed until they metamorphosed, a dose-dependent increase in mortality and number of animals showing malformations was observed at exposure concentrations as low as 17.2 pM PCB 126 (Gutleb et al., 1999) thus giving a completely different picture of possible toxicity of PCB 126 for amphibians than the 96-h FETAX.

Tadpole growth and development is known to be density dependent (Werner, 1986; Scott, 1994). Factors that influence the number of tadpoles within an experimental unit once the experiment started such as either mortality or fast metamorphosis do not only change the animal density within the water volume of the experimental unit but thereby also influence the development of the remaining experimental animals. For example high mortality at the beginning of an experiment may increase the body weight of the remaining animals by increasing water volume and food per individual tadpole.

The objective of the present study was to further improve the protocol of the prolonged-FETAX in a way to overcome the interference of mortality or fast rate of metamorphosis on the remaining animals by changes of animal densities that will interfere with compound related effects the test should be able to pick up. A protocol was developed in which the water volume is kept constant throughout the whole experimental period relative to the number of animals remaining in the experimental group. This new protocol was applied to Clophen A50, a technical PCB mixture that resembles the congener pattern present in wildlife, the planar congener PCB 77, that is easily metabolised, and to sediment extracts that have been shown to cause proliferation in the thyroid hormone dependent T-Screen (Gutleb et al., 2005) and to influence amphibian metamorphosis in the Synchronized Amphibian Metamorphosis Assay (Gutleb et al., subm.).

## ANIMALS, MATERIALS AND METHODS

### *Chemicals*

The technical PCB mixture Clophen A50 was a kind gift of Jan Boon (NIOZ, Den Burg, The Netherlands). PCB 77 (99% purity) was obtained from Promochem (Wesel, Germany). Human chorionic gonadotropic hormone (hCG) was obtained from Organon (Oss, The Netherlands). All other chemicals used throughout the experiments were of analytical grade and obtained from Merck (Darmstadt, Germany).

*Preparation of sediment extracts*

Two sediment samples were from known polluted sites in the Netherlands, namely the Westerschelde close to Terneuzen, and the Dommel close to Eindhoven (Vethaak et al., 2002). In addition sediment samples were collected from two locations on a small channel close to Hoogeveen, where malformed amphibians have been observed in the field. These two sediments were characterized by differences in the concentrations of chemical pollutants found (Torenbeek, pers. comm.) and are labelled as Hoogeveen I and Hoogeveen II, of which the later was taken downstream and had lower concentrations of several polyaromatic hydrocarbons compared to Hoogeveen I. Apolar fractions of sediment were obtained as previously described (Legler et al., 2002). Briefly portions of sediment (10 x 2g) were vortexed (1 min) after addition of 2 ml of acetone and allowed to stand for 10 minutes prior to vortexing (1 min) again. A mixture (4 ml) of hexane/dieethylether (Hx-DEE, 9:1) was added and after vortexing (1 min) samples were centrifuged for 2 minutes (400g) and the upper layer of all ten parallel extracts was transferred into pre-washed glass tubes. This step was performed 3 times after which 200 µl Hx-DEE was added and the upper layer was collected again. Solvent was evaporated at 30 °C under a gentle stream of nitrogen. Prior to complete evaporation the ten samples were quantitatively pooled in a single vial, slightly warmed and kept under a gentle stream of nitrogen until dryness. Hexane (500 µl) was added and the sample was filtered over a 1 g $Na_2SO_4$ column, evaporated and taken up into 20 µl DMSO resulting in an apolar fraction.

*Animals, breeding and housing*

All experiments were performed at the Toxicology Section, Wageningen University and Research Centre. Adult African clawed frogs (*Xenopus laevis*) were obtained from the Department for

Experimental Zoology, Catholic University of Nijmegen. All experimental animals were kept at a 12:12 photoperiod schedule. Adult *X. laevis* were maintained in aquaria with constantly filtered and aerated water at 20 °C. Details of breeding conditions have been reported previously (Fort and Bantle, 1990; Gutleb et al., 1999; 2000). Developmental stage of embryos and tadpoles was scored according to Nieuwkoop and Faber (1975).

*Experimental protocol*

Details of methods applied throughout the FETAX assays have been reported previously (Gutleb et al., 1999). DMSO was used as a solvent throughout all FETAX exposures at 0.5% in the water phase. The exposure medium was renewed every day. The FETAX and prolonged-FETAX exposures always were performed in quadruplicate (4x25 embryos starting at NF stage 8-11) at 24 °C and lasted for 96-h when embryos had reached NF stage 46.

Experiment 1: Embryos were exposed to PCB 77 (0.03 nM, 3 nM, 300 nM, 30,000 nM) or to DMSO in quadruplicate.

Experiment 2: Embryos were exposed to apolar sediment extracts (equivalent to 250 mg sediment in 10 ml exposure medium) or to DMSO in quadruplicate.

Animals from the duplicates that were ad random assigned for the FETAX were anaesthetised in ice water and thereafter fixed in formalin (4%) for microscopic examination after 96-hours when animals have reached NF stage 46. For the prolonged-FETAX the surviving tadpoles of two FETAX duplicates (NF stage 46) were placed in aquaria of 20x20x30 cm (400 ml water/animal). Aquaria were checked daily for the presence of dead animals and tadpoles found dead were fixed and scored for malformations (Bantle et al., 1991). Tadpoles were fed a diet consisting of 500 g dried nettle powder (Jacob Hooy, Limmen, The Netherlands), 5 g coffee-creamer (Friesland Dairy Foods, Leeuwarden, The Netherlands), 5 g agar

granulated and 5 g yeast extract (both from Becton Dickinson, Cockeysville, USA). The powder (200 mg) was mixed with tap water to get a viscous mixture, which was added once a day to the aquaria. Water from all exposure experiments was charcoal-filtered prior to disposal. Room temperature in experiment 1 was set to 21 °C whereas in experiment 2 the temperature was set to 24 °C throughout the complete experimental periods.

Animals that reached metamorphic NF stage 65/66 were anaesthetised in icewater and thereafter sacrificed by cervical transection, weighed, scored for malformations and wrapped in aluminiumfoil and stored at −20 °C. Water volume was continuously kept constant per animal by removing 400 ml per animal taken out of an aquarium. At the end of the 115-day experimental period, the remaining tadpoles were anaesthetised in ice-water, sacrificed by cervical transection and their developmental stage was scored according to (Nieuwkoop and Faber, 1975). The results are presented in three different ways starting simply by comparing percentages of animals that passed metamorphosis as defined by reaching NF stage 65. NF stage 65 was chosen as the final stage for our goal as the disappearance of the tail remnants that mark the difference between NF stage 65 and NF stage 66 can vary over several days and is not dependent on changes of thyroid hormone anymore. Secondly tadpoles are ranked into three categories according to their development, namely stages before thyroid hormone dependency (NF stages <56; early metamorphic), peak thyroid hormone dependent stages (NF stages 56–60, metamorphic) and late metamorphic stages (NF stage 61-65) that show decreasing thyroid hormone levels (Etkin, 1932). Thirdly penalty points are accredited to each animal per group based on the number of developmental stages possibly lacking until finalising metamorphosis. Animals are accredited 0 penalty points when they had reached NF stage 65, 1 for NF stage 64 and so forth.

*Data analysis*

All data are reported as means ± standard error of the mean (SEM). Differences between means were tested with one-way ANOVA respectively Kruskal-Wallis H-Test where appropriate. The acceptance level was set at $P<0.05$. Statistical analyses were performed using SPSS/PC+, version 6.0 (SPSS Inc., Chicago, IL, USA). The scores for the animals that passed metamorphosis and the stages of tadpoles that did not pass metamorphosis were compared between treatments by $\chi^2$ contingency analysis using GraphPad Prism 4 (San Diego, CA, USA).

## RESULTS

Experiment 1:

FETAX: After 96-h exposure to any of the PCB 77 concentrations (0.03 nM, 3 nM, 300 nM, 30,000 nM) low mortality and rate of malformations were observed (< 10%) that were not dose-dependent and no significant effects on growth were observed with mean body lengths for all groups ranging from 7.9 – 8.2 mm (one-way ANOVA, $P>0.05$).

prolonged-FETAX: Animals in one of the two groups that has been exposed to 3 nM PCB 77 were terminated during the experimental period after having suffered from a decrease in water quality as a fungus affected the detritus in the aquarium. Results from this group are not included in the tables and figures. In all the other groups mortality was less than 15% and no malformations occurred until the end of the experiment (day 115). Body weight at NF stage 65/66 and duration of larval period until reaching NF stage 65 was not significantly different between any of the groups (Table 1).

**Table 1.** Number of animals (n) of the total number of surviving animals (N) that had reached NF stage 65 after 115 days, average body weight (mg ± SEM) and length of larval period (days ± SEM) of the animals that reached NF stage 65 after early exposure during the first 96-hours (NF stage 13-46) to PCB 77.

| PCB 77 (nM) | n/N | body weight (mg ± SEM) | larval period (days ± SEM) |
|---|---|---|---|
| 0 | 31/48 | 180 ± 9 | 93.2 ± 3 |
| 0.03 | 31/46 | 167 ± 7 | 87.2 ± 3 |
| 3# | 15/22 | 205 ± 6 | 96.2 ± 2 |
| 300 | 19/46 | 215 ± 14 | 93.3 ± 3 |
| 30,000 | 17/45 | 168 ± 8 | 93.3 ± 3 |

Statistical evaluation (body weight, larval period) was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Bonferroni's test that controlled Type I errors; # one group of the duplicates was terminated earlier due a decrease in water quality (data not included)

Early life-stage exposure to 300 nM and 30,000 nM PCB 77 resulted in significant decreased percentage of animals that passed metamorphosis compared with control groups 115 days after exposure had been ceased ($\chi^2$=36.85; d.f.=4; $P$<0.05), whereas after 60 days ($\chi^2$=12.65; d.f.=4; $P$>0.05), 90 days ($\chi^2$=12.80; d.f.=4; $P$>0.05), and 100 days ($\chi^2$=19.48; d.f.=4; $P$>0.05) no significant differences were found (Figure 1).

In the groups exposed to 3 nM PCB 77 the percentage of tadpoles that were still early metamorphic (< NF stage 56) on day 115 was significant different compared with control groups ($\chi^2$=17.83, d.f.=4; $P$<0.05). Exposure to 300 nM and 30,000 nM PCB 77 resulted in significantly increased percentages of metamorphic tadpoles ($\chi^2$=21.18, d.f.=4; $P$<0.05) (NF stages 56-60), whereas no such differences compared with control groups was found for late metamorphic tadpoles for any exposure concentration (NF stages 61-64) after 115 days in exposed groups ($\chi^2$=19.20, d.f =4; $P$>0.05).

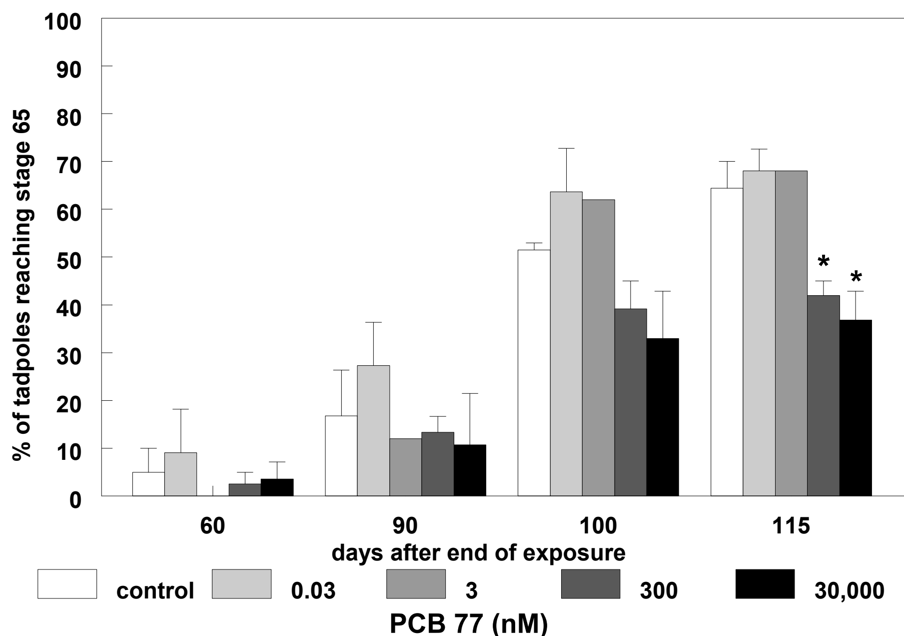


**Figure 1.** Percentage of tadpoles reaching NF stage 65 after exposure of NF stages 8-46 to PCB 77 (nM) during 96-h, followed in their further development without any additional exposure according to the prolonged-FETAX protocol.

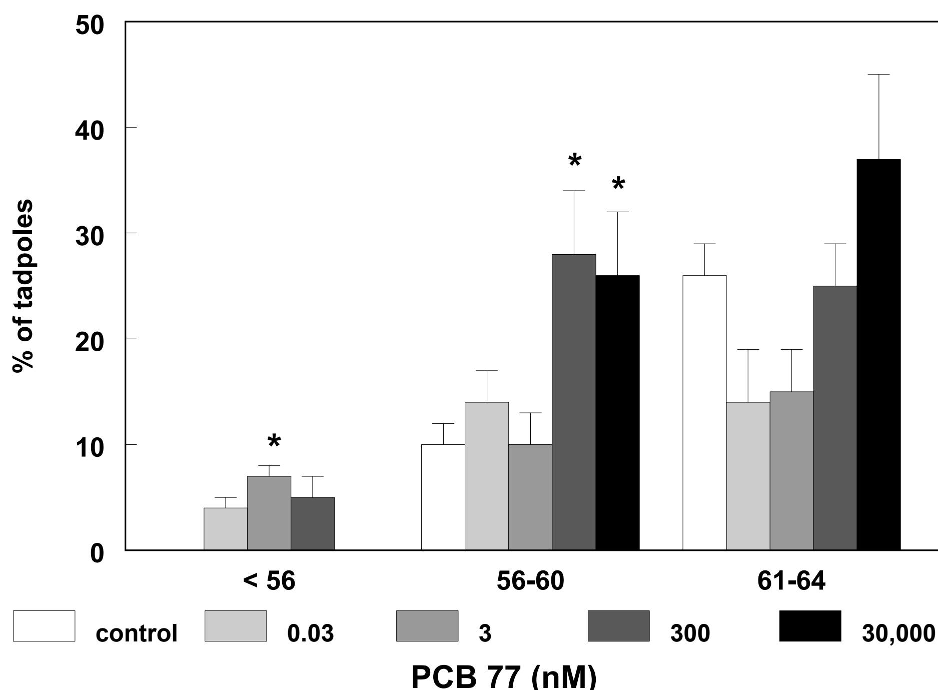


**Figure 2.** Distribution of tadpoles that did not metamorphose within the experimental period of 115 days into three different developmental categories (Etkin, 1932). The animals were exposed to PCB-77 (nM) according to the prolonged-FETAX protocol during 96-h from NF stage 8 to NF stage 46, and followed in their further development without any additional exposure. The percentage of tadpoles that did finish metamorphosis in 115 days was indicated in Figure 1.

The number of penalty points awarded to prolonged-FETAX exposed tadpoles at the end of the experimental period converting developmental stages into penalty points starting with NF stage 65 = 0, NF stage 64 = 1 and so forth differed significantly from controls for the groups exposed to 300 nM PCB 77 or higher (Kruskal Wallis H-Test, $P$>0.05) (Figure 3).

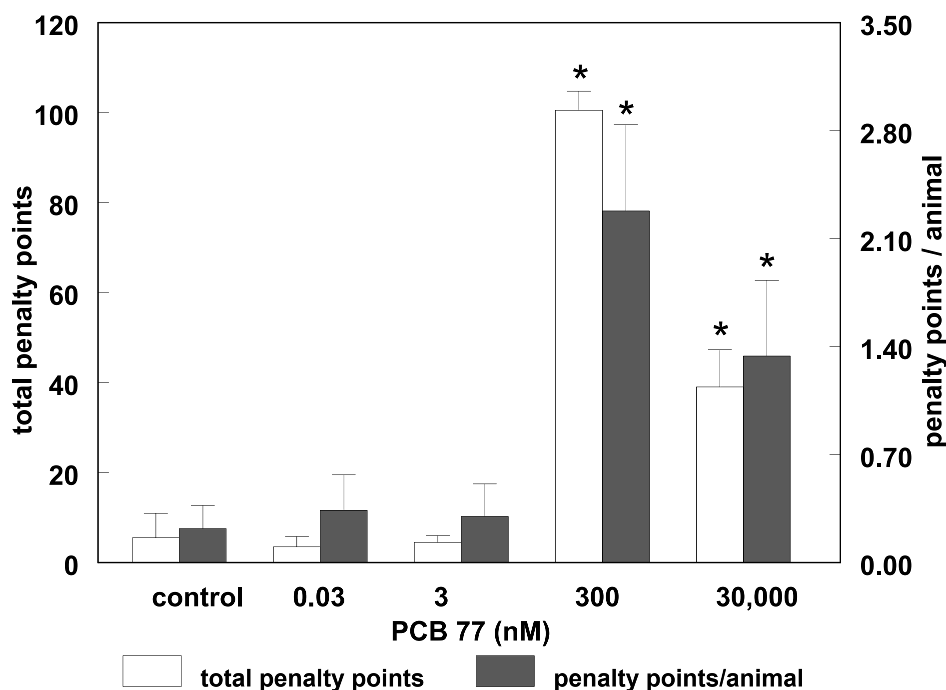


**Figure 3.** Total penalty points/group and mean penalty points/animal at the end of the 115-day experimental period. Animals reaching NF stage 65 were counted as 0, NF stage 64 was 1 and so forth. Statistical evaluation was made by Kruskal Wallis H-Test, * $P$<0.05

Experiment 2:

FETAX: After 96-h exposure to apolar sediment extracts (exposure equivalent to extract of 250 mg sediment/10 ml) low mortality and low rate of malformations were observed (< 10%) both in control groups and in apolar sediment extracts exposed groups. Growth in the 96-hour exposure period was not affected in any group (mean body length ranging from 7.9 – 8.2 mm) (one-way ANOVA, $P$>0.05).

prolonged-FETAX: Animals in one group of the duplicates that had been exposed to apolar sediment extracts from Hoogeveen I were terminated during the experimental period after having suffered from a decrease in water quality as a fungus affected the detritus in the aquarium. Results from this group are not included in the tables and figures. Mortality in any of the other exposure groups was less than 15% and no malformations occurred in any of the groups until day 115. Apolar sediment extracts from Terneuzen significantly decreased body weight (one-way ANOVA, $P<0.01$) at NF stage 65/66 compared with the control groups (Table 2). Apolar sediment extracts from both locations in Hoogeveen resulted in a trend to decreased body weight at NF stage 65/66 (one-way ANOVA, $P<0.1$) (Table 2).

Apolar sediment extracts from Terneuzen significantly increased duration of larval period (one-way ANOVA, $P<0.05$) compared with the control group (Table 2). Duration of larval period was longer in all other sediment-exposed groups compared with the control groups although not significant (one-way ANOVA, $P>0.05$).

Early life-stage exposure to apolar sediment extracts from Dommel and Terneuzen resulted in significant reduced percentages whereas exposure to apolar sediment extracts from both locations in Hoogeveen resulted in significant increased percentages of animals that passed metamorphosis after 90, 100 and 115 days (resp. $\chi^2=67.64$, $\chi^2=60.96$, and $\chi^2=52.99$; d.f.=4; $P<0.05$). Similar to experiment 1 variation between duplicates was low in the control group and higher in the exposed groups (Figure 4).

**Table 2.** Number of animals (n) of the total number of surviving animals (N) that had reached NF stage 65 after 115 days, average body weight (mg ± SEM) and length of larval period (days ± SEM) of the animals that reached NF stage 65 after early exposure during the first 96-hours (NF stage 13-46) to sediment extracts (equivalent to 250 mg sediment in 10 ml exposure medium)

| sediment extract | n/N | body weight (mg ± SEM) | larval period (days ± SEM) |
|---|---|---|---|
| control | 29/46 | 234 ± 14 | 76.6 ± 6 |
| Dommel | 23/47 | 223 ± 14 | 81.2 ± 3 |
| Terneuzen | 20/48 | 175 ± 11** | 87.8 ± 2* |
| Hoogeveen I# | 18/23 | 184 ± 15(*) | 83.8 ± 3 |
| Hoogeveen II | 38/48 | 190 ± 8(*) | 80.1 ± 3 |

Statistical evaluation (body weight, larval period) was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Bonferroni's test that controlled Type I errors, (*) $P<0.1$; * $P<0.05$; ** $P<0.01$; # one group of the duplicates was terminated earlier due a decrease in water quality (data not included)

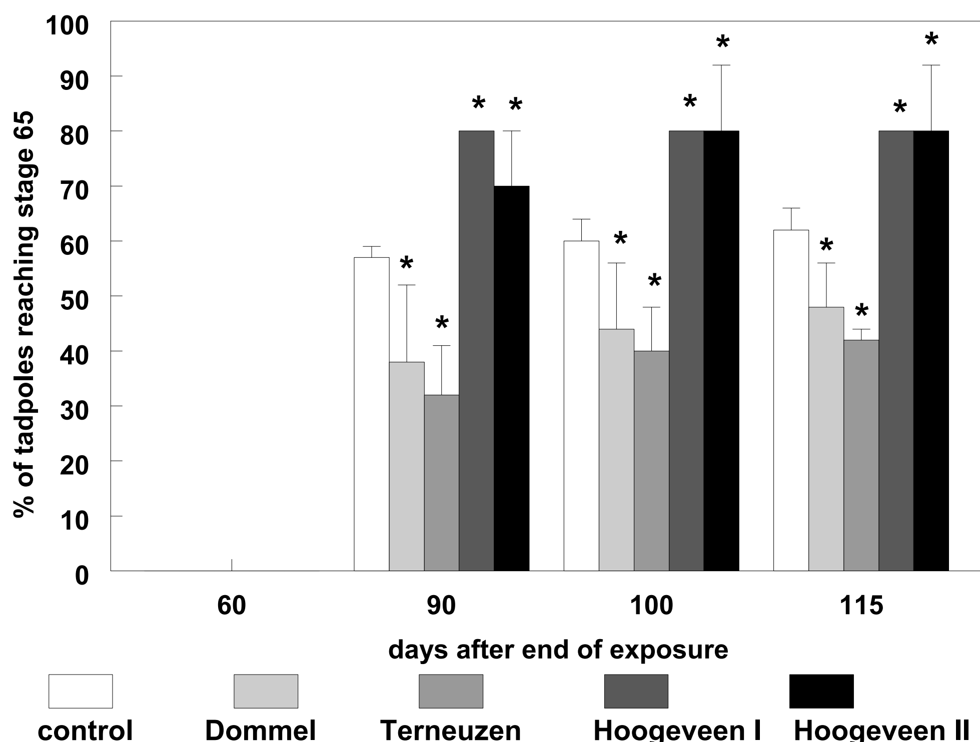


**Figure 4.** Percentage of tadpoles reaching NF stage 65 after having been exposed to apolar sediment extracts (equivalent to 250 mg sediment in 10 ml exposure medium) during 96-h from NF stage 8 to NF stage 46, followed in their further development without any additional exposure according to the prolonged-FETAX protocol.

Of the animals that did not finish metamorphosis in the groups exposed to apolar sediment extracts from Dommel and Terneuzen the highest percentage was halted in NF stage 56-60, and to a lesser extend in NF stages ≤55. These percentages were significantly higher than in the control group after 115 days ($\chi^2$=13.31, d.f.=4; $P$<0.05) whereas the percentage was significantly decreased for the Hoogeveen I exposed group ($\chi^2$=4.88, d.f.=4; $P$<0.05). Exposure to apolar sediment extracts from Hoogeveen I significantly decreased the percentage of late metamorphic tadpoles (NF stage 61-64) after 115 days ($\chi^2$=11.15, d.f.=4; $P$<0.05) (Figure 5).

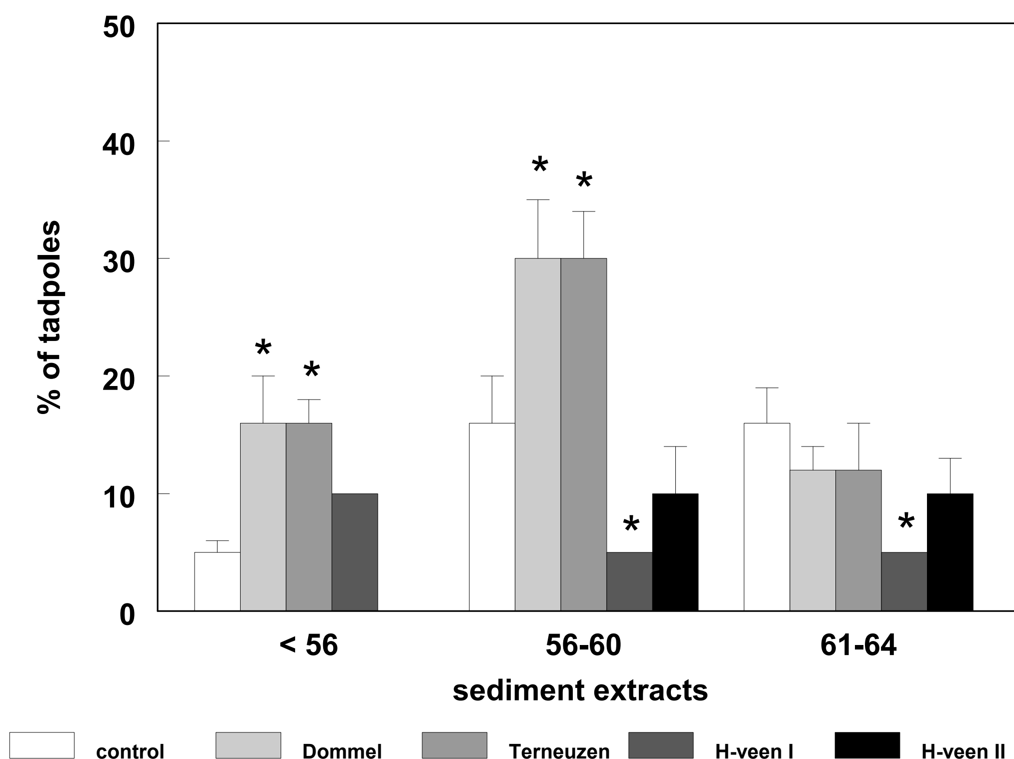


**Figure 5.** Distribution of tadpoles that did not metamorphose within the experimental period of 115 days, expressed as % of total number of tadpoles divided into three different developmental categories (Etkin, 1932). The animals were exposed to apolar sediment extracts (equivalent to 250 mg sediment in 10 ml exposure medium) according to the prolonged-FETAX protocol during 96-h from NF stage 8 to NF stage 46, and followed in their further development without any additional exposure. The percentage of tadpoles that did finish metamorphosis in 115 days was indicated in Figure 4.

The number of penalty points awarded to froglets and tadpoles at the end of the experimental period converting developmental stages into penalty points starting with NF stage 65 = 0, NF stage 64 = 1 and so forth differed significantly for groups exposed to apolar sediment extracts from Dommel and Terneuzen (Kruskal Wallis H-Test, *P*>0.05) (Figure 6).

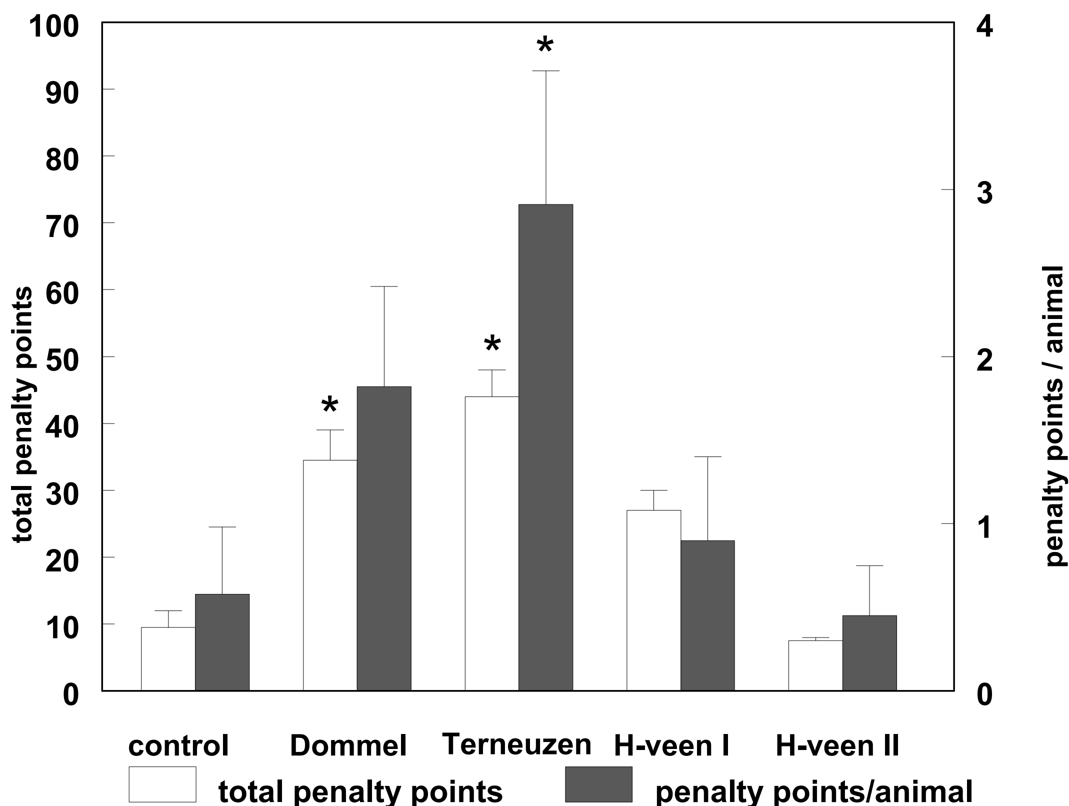


**Figure 6.** Total penalty points / group and mean penalty points / animal at the end of the 115 day experimental period. Animals reaching NF stage 65 were counted as 0, NF stage 64 was 1 and so forth. Statistical evaluation was made by Kruskal Wallis H-Test, * *P*<0.05

## DISCUSSION

The developmental toxicity of PCB 77 and of apolar sediment extracts from polluted sites in the Netherlands was evaluated with the FETAX and with the further developed prolonged-FETAX. The current study was undertaken to evaluate possible delayed effects of

the less persistent PCB 77 as in an earlier study delayed effects for the dioxin-like and very persistent PCB 126 were found in a similar experimental approach. Additionally this is the first attempt to study long-term effects of early life stage exposure to diluted sediment extracts to test whether exposure levels in the natural environment could be high enough to result in disturbed development.

The mortality and malformation rates in the control, PCB 77 and sediment extract exposure groups always were below the rates that are considered acceptable according to ASTM guidelines for the FETAX (ASTM, 1991). None of the tested compounds resulted in inhibition of growth during the 96 hours of exposure, the most sensitive parameter of the FETAX (Dawson and Bantle, 1987). This means that solely based on results from FETAX results it would be concluded that both PCB 77 and the apolar sediment extracts likely pose no risk to amphibians. The results of the prolonged-FETAX performed within this study reveal that this conclusion no longer holds.

Also in the prolonged-FETAX mortality always was less than 15% and no malformations occurred. No delayed effects of early life-stage exposure to PCB 77 were found on either body weight or duration of larval period resembling earlier results for PCB 126 (Gutleb et al., 1999). The increase in the room temperature from 21 °C (experiment 1) to 24 °C (experiment 2) resulted in a shorter experimental period without leading to increased malformation percentages. Contrary to PCB 126, exposure to PCB 77 in the concentration range from 0.03 nM up to 30,000 nM did not result in increase of malformations although concentrations covered similar orders of magnitude calculated on basis of published TEF values for the two congeners (van den Berg et al., 1998). PCB 77 is much more readily metabolized than PCB 126 and although to our knowledge nothing has been published on the metabolic capacity of tadpoles for PCBs, metabolization and excretion of PCB 77 resulting

in decreasing body burden could explain the observed differences in long-term toxicity. There is some evidence for such a conversion and excretion as oral exposure of tadpoles to increasing concentrations of PCB 77, was not reflected by an increase in body burdens (own unpubl. results).

Exposure of embryos for 96 hours to PCB 77 at concentrations of 300 nM resulted in a significant decreased percentage of metamorphosed froglets at various observation times throughout the experimental period, giving evidence for a perturbation of thyroid hormone dependent metamorphosis. The significantly higher number of tadpoles that remained in the thyroid hormone peak dependent early metamorphic NF stages 56-60 and the significantly increased number of penalty points per animal and per group are further indications for negative effects of early life stage exposure on thyroid hormone homeostasis. PCB 126 exposure in a prolonged-FETAX had resulted in 24% lowered $T_4$ levels at concentrations as low as 7.7 pM at the end of the experimental period more than 60 days after exposure has been ceased (Gutleb et al., 1999). Possible mechanisms of thyroid hormone alteration by PCB 77 include both the transport and the metabolization of thyroid hormones (Brouwer et al., 1998).

Decreased body weight of NF stage 65/66 froglets after exposure to apolar sediment extracts from Terneuzen and both sites at Hoogeveen can at present not be attributed to specific compounds. These sediments are heavily polluted with high concentrations of a wide range of compounds including wastewater effluents and industrial compounds such as brominated flame-retardants, or fluoranthene and pyrene (Gutleb et al., subm.). Although a greater percentage of the animals exposed to the Hoogeveen extracts had passed metamorphosis, the larval period of the successful animals was not shorter (Table 1) which could have explained a lower froglet weight. Furthermore the sediment extracts tested in the prolonged-

FETAX altered thyroid hormone dependent cell growth in the T-Screen. Interestingly, the apolar extracts from the Dommel decreased and those from both sites in Hoogeveen increased cell growth significantly (Gutleb et al., 2005), which is in accordance with the *in vivo* results found in this study. Moreover sediments from the Netherlands have recently been shown to contain principles with thyroid-hormone mimicking ability, in this case displacing $T_4$ from transthyretin in the $T_4$-TTR assay (Houtman et al., 2004). Alterations, both in the form of increased or decreased levels or of the ratios of $T_3$ and $T_4$ will affect the thyroid hormone dependent regulated timing of amphibian metamorphosis (Bray and Sicard, 1982), which consecutively will result in changes in body weight of metamorphosed froglets.

The fact that exposure to apolar sediment extracts from Dommel and Terneuzen resulted in less animals that did complete metamorphosis, in a longer larval period for those froglets that had passed metamorphosis, an increased percentage of animals still in thyroid hormone dependent early metamorphic NF stages 56-61, and a significant increased number of penalty points for these animals are in accordance with the decrease of thyroid hormone dependent cell growth reported for the cells exposed to these sediment extracts in the T-Screen (Gutleb et al., 2005). The same is true for the apolar sediment extracts from Hoogeveen I and Hoogeveen II that resulted in more animals passing metamorphosis within the experimental period of 115 days, and a lower number of animals in early developmental stages on day 115 as reflected by the number of awarded penalty points, that parallels the increase of thyroid hormone dependent cell growth cells exposed to the same extracts in the T-Screen (Gutleb et al., 2005).

Thyroid hormone is crucial in embryonic development and growth, and controls metamorphosis by regulating the expression of a cascade of genes (Shi, 1994). Without a thyroid hormone surge

shortly before the onset of metamorphosis amphibian larvae are not able to reach late-metamorphic NF stages (≥ NF stage 61) at which thyroid hormones are no longer necessary to complete metamorphosis (Gudernatsch, 1912; Etkin, 1932). Any increase of thyroid hormone activity present in the tadpoles due to uptake of thyroid hormone mimicking compounds will result in a shorter larval period and in decreased overall percentages of tadpoles in early metamorphic and metamorphic stages respectively will be reflected by low mean and total penalty points whereas the opposite is true for compounds with antagonistic effects. The differences of percentages of tadpoles in early-, metamorphic and late metamorphic stages that were almost in all cases significant are a further indication of alteration of thyroid hormone homeostasis.

It is not yet clear what the ecological relevance will be of the observed alterations of tadpole development resulting from early life stage exposure such as lowered percentage metamorphosed tadpoles and longer or shorter duration of metamorphosis on wild-living amphibian populations. But even a shorter duration of metamorphosis could be adverse when this interferes with the availability of adequate food resources for the terrestrial carnivorous froglets in temperate climates. A longer duration of metamorphosis and smaller tadpole body size is correlated with increased predation risk (Travis, 1983; Richards and Bull, 1990). It cannot be excluded that metamorphosing at a larger body size increases survival of terrestrial juveniles and their size at first reproduction, and thereby, increase individual and in some cases, population fecundity (Berven and Gill, 1983; Semlitsch et al., 1988; Scott, 1994).

## CONCLUSIONS

Exposure of *Xenopus laevis* embryos for 96-hours to PCB 77 resulted in delayed long-term effects on the percentage of metamorphosed froglets and in the same exposure regime sediment exposure of amphibian embryos resulted in changes of body weight, length of period until metamorphosis and percentage of metamorphosed froglets. In parallel experiments these compounds did not cause any effect whatsoever in the classical FETAX. Therefore the prolonged-FETAX is an important additional tool to assess developmental toxicity of single compounds as well as of complex mixtures of environmental relevance. Developmental effects in the FETAX and prolonged-FETAX are not only relevant to indicate toxicity for amphibians, but also for other vertebrates as amphibians often are used as model-organisms for other vertebrate species.

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# Intermezzo

## Latex laboratory-gloves: an unexpected pitfall in amphibian toxicity assays with tadpoles

*[Environ. Toxicol. Pharmacol. 2001, 10, 119-121]*

Multiple oedema and malformations in tadpoles resulting from embryonic exposure to PCB 126 (prolonged-FETAX, Chapter 2)

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Environmental Toxicology and Pharmacology 10 (2001) 119–121

ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY ETAP

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# Latex laboratory-gloves: an unexpected pitfall in amphibian toxicity assays with tadpoles

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**Abstract**

This study examined the unexpected toxic effects of protective latex laboratory gloves on developing amphibians. Mortality after exposure to rinsing water from the outside of the gloves was observed in *Xenopus laevis* and *Rana temporaria*, with *R. temporaria* being more sensitive. This phenomenon was further confirmed using the microtiter-version of the Microtox-Assay, an in vitro assay for general toxicity. Latex gloves from the specific brand used in the experiment, in which the toxicity to tadpoles was observed for the first time, showed the highest toxicity of all materials and brands tested. Due to the high responsiveness of amphibian tadpoles to latex-glove contaminated rinsing water, special care is necessary when cleaning aquaria during toxicological experiments with amphibians as otherwise results may be biased. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* Gloves; Latex; Frog; *Rana*; *Xenopus*

## 1. Introduction

Amphibian larvae are frequently used for toxicity testing such as in the FETAX-Assay (Bantle and Sabourin, 1991; ASTM, 1991), in the prolonged-FETAX-Assay (Gutleb et al., 1999) or effects of xenobiotics on later developmental stages are studied (Fioramonti et al., 1997; Gutleb et al., 2000). In experiments covering the total length of amphibian metamorphosis, cleaning the larval tanks to remove debris is absolutely necessary. Wearing protective gloves during cleaning procedures is essential not only to prevent contamination of the experimentator, but also for hygienic purposes.

In a prolonged-FETAX-Assay (Gutleb et al., 1999), acute mortality without preceding symptoms was observed in the untreated control group (47.5%) and in the DMSO treated vehicle control group (45.5%). *Xenopus laevis* tadpole mortality occurred shortly after staff members, using latex-gloves, cleaned the aquarium of the respective group.

Even though literature had been screened for toxicological studies with tadpoles we were not aware of any study mentioning this effect. Therefore we decided it was necessary to collect more information on the potential threat of protective gloves for amphibian larvae during in vivo experiments. In order to obtain information on the best type of material of gloves for in vivo experiments we tested the toxic potential of four types of latex gloves, and one type of vinyl, respectively nitril gloves using the microtiter version of the classical Microtox-Assay with *Vibrio fischeri* (Hamers et al., 2001).

## 2. Animals, materials and methods

All experiments were performed at the Toxicology Section of Wageningen University. Adult African clawed frogs (*X. laevis)* were obtained from the Department for Experimental Zoology, Catholic University of Nijmegen and were maintained in aquaria with constantly filtered and aerated water at 20 °C at a 12:12 photoperiod. Animals were fed with beef heart or commercial trout food (Provimi Agra, Zwolle, The Netherlands) twice weekly. Viable gametes were obtained as previously described (Gutleb et al., 1999). Common

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frogs *(Rana temporaria)* were collected on 30 March, 1995 near Maria Saal, Carinthia, Austria (46° 38′ 30″, 14° 22′ 30″E) before they entered a pond for breeding. Details on the transport of *R. temporaria* and housing conditions for both species have previously been described (Gutleb et al., 1999). The developmental stage of tadpoles was scored according to Nieuwkoop and Faber (1975) and Gosner (1960) for *X. laevis* and *R. temporaria* respectively.

The outer side of 10 latex gloves (size L, Becton-Dickinson) or 10 vinyl gloves (size L, Becton-Dickinson) was soaked in 3-l tap water for 24 h at 20 °C. These stock solutions were further diluted down to a maximum of 1:900 using tap water as a diluent. Ten tadpoles of stage 51/52 (*X. laevis*) and stage 30 (*R. temporaria*), respectively were placed in aquaria (10 × 10 × 10 cm, total volume of water was 700 ml). Mortality of tadpoles was scored after 12 h of exposure.

Exposure medium for the microtiter version of the classical Microtox-Assay was prepared by stirring 20 ml 2% NaCl solution with the middle finger of the glove (for the different brands see Table 1) for 1 min in a 100-ml beaker. Details on the 96-wells plate assay using the bacterium *V. fischeri* are published elsewhere (Hamers et al., 2001). Luminescence in the wells was analysed in duplicate after 30 min (Ribo and Kaiser, 1983) in a luminometer (Labsystems Luminoskan RS).

## 3. Results

Tadpoles of both species showed a dose dependent mortality when exposed to increasing concentrations of latex contaminated water (Fig. 1). All *X. laevis* tadpoles were dead after 12 h when exposed to dilutions of latex–glove–water of 1:350 or less and 50% mortality was calculated for a dilution of 1:425, which corresponds to 0.235% latex–water present or one glove in 128 l of water. *R. temporaria* tadpoles were even more sensitive with 100% mortality in dilutions of the latex–water of 1:600 or less. This corresponds to 0.15% latex–water or one glove in 195 l water. Contrary to the findings for latex–water no mortality of *X. laevis* tadpoles was observed in rinsing water of vinyl-gloves at dilutions lower than 1:4 with 100% mortality when exposed to dilutions of 1:3 or less. Surviving tadpoles showed no symptoms whatsoever.

Table 1
Inhibition of luminescence in the microtiter version of the classical microtox-assay by rinsing water from the outside of six different gloves (control = 100%)

| Material | Pure solution | 1:2 | 1:4 | 1:8 |
|---|---|---|---|---|
| B&D latex[a] | 2 | 40 | 58 | 97 |
| Maxxim latex | 46 | 88 | 80 | 102 |
| Klinion latex | 51 | 87 | 97 | 100 |
| Romed latex | 58 | 92 | 100 | 98 |
| Romed vinyl | 77 | 98 | 99 | 99 |
| Romed nitril | 84 | 99 | 99 | 96 |

[a] Used for the experiments with the tadpoles.

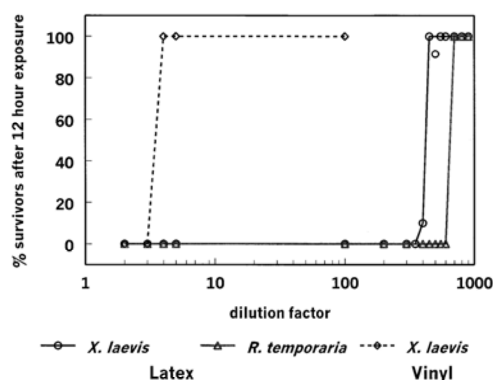


Fig. 1. Survivors (as % of 10 animals/group) of *Xenopus laevis* and *Rana temporaria* tadpoles exposed to soaking solutions from the outside of gloves.

Luminescence of *V. fischeri* after 30 min of incubation with the glove rinsing solution was decreased to 2% for the most toxic latex glove to 83.6% of the control values for the nitril-glove, respectively (Table 1). The latex-glove used in the experiment with tadpoles was also the most toxic one in this assay (Table 1).

## 4. Discussion

The present study was undertaken to evaluate the toxic potency of protective laboratory gloves made from different materials for amphibians, as sudden mortality was observed in *X. laevis* tadpoles, which were exposed to latex gloves during routine cleaning procedures of their aquarium (Gutleb et al., 1999). As hardly anything was found after thorough searching of information on toxicity of gloves to amphibians we feel obliged to publish these results in order to prevent unnecessary mortality and waste of experimental animals.

*R. temporaria*, a common amphibian species on the European continent, was found to be even more sensitive to latex-glove rinsing water than *X. laevis* with both species showing mortality up to high dilutions. The latex gloves used for the exposure of the tadpoles were those that also showed the highest effect in the microtiter version of the Microtox-Assay. Lethal effects of latex-gloves on *X. laevis* tadpoles have been described earlier in a note to the editor by Sobotka and Rahwan (1994) and it is not the powder used for the lubrication of the inner side of the gloves, which is responsible for the observed mortality. A wide range of compounds is

added to latex during the fabrication process to ensure good fabrication and usage properties. The toxic effect of latex-glove extracts for humans can be attributed to compounds such as dithiocarbamate accelerators and thioureas leaching out of the latex (Nakamura et al., 1990). In a Dutch report, it was described that 82% of water extracts from different brands of latex gloves were shown to be cytotoxic in a battery of four different in-vitro tests (cell growth, LDH leakage, ATP depletion, Agar overlay test) (Geffen et al., 1994). Unfortunately this is an internal report and not internationally accessible.

## 5. Conclusion

Up to now latex-glove born compounds respective the chemicals added to improve the properties of the product have not been tested in respect to amphibian toxicity. Due to the high responsiveness of both species, but especially of *R. temporaria* to latex-glove contaminated rinsing water, special care is necessary when cleaning aquaria during exposure experiments with amphibians. Both vinyl- and nitril gloves are far better to use than latex-gloves. It is advised to test gloves with an in vitro assay for general toxicity before using them in water in which tadpoles are kept in order to prevent sudden loss of experimental animals.

## Acknowledgements

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# 4

# Effects of oral exposure to polychlorinated biphenyls (PCBs) on the development and metamorphosis of tadpoles from two amphibian species (*Xenopus laevis* and *Rana temporaria*)

[*Science Total Environ. 2000, 262, 147-157*]

*Xenopus laevis* tadpoles
NF stage 61/62 (up) and NF stage 65 (down)

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# Effects of oral exposure to polychlorinated biphenyls (PCBs) on the development and metamorphosis of two amphibian species (*Xenopus laevis* and *Rana temporaria*)

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**Abstract**

This study examined the effects of polychlorinated biphenyls (PCBs) on development of families of amphibians using the African clawed frog (*Xenopus laevis*) and the European common frog (*Rana temporaria*). Amphibians were orally exposed to the technical PCB-mixture Clophen A50 or to the non-ortho-3,3′,4,4′,5-CB congener (PCB 126) either for a 10-day period or until metamorphosis. Occurrence and rate of malformations, mortality, period until metamorphosis and thyroid hormone levels were measured. Mortality increased in a dose-dependent manner, as did the rates of malformation. Time until metamorphic transformation was prolonged and the weight of froglets was increased. Although not statistically significant, thyroid hormone levels were also lowered. PHAHs such as PCBs may affect important aspects of amphibian fitness and may influence amphibian reproductive success. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Frog; PCBs; Larval development; Thyroid hormone; Amphibian decline

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## 1. Introduction

In addition to habitat destruction and increased UV-radiation, the current decline of amphibian populations may be caused by ubiquitous, persistent organic substances belonging to the group of polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorinated biphenyls (PCBs) (Blaustein and Wake, 1990; Wake, 1991; Griffiths and Beebee, 1992). Amphibians potentially are exposed to toxic compounds via several routes. The presence in most species of a highly permeable skin, the development of their eggs and larvae in the water and their changing position in the food web from herbivorous algae eating tadpoles to carnivorous adults favours uptake of pollutants. Polychlorinated biphenyls (PCBs) have been shown to alter early embryonic and larval development of amphibians at environmentally realistic concentrations (Gutleb et al., 1999).

In mammals and birds, PCBs are able to disrupt thyroid hormone homeostasis by influencing metabolism (Brouwer et al., 1988; Murk et al., 1994), and after metabolisation into hydroxylated metabolites by disrupting the plasma transport of thyroxin ($T_4$) (Brouwer, 1989; Brouwer et al., 1998). Thyroid hormone is important in development and in many regulatory functions in all vertebrate species; in amphibians there is an additional important function of thyroid hormone. Without a thyroid hormone surge shortly before the onset of metamorphosis, amphibian larvae are not able to reach the post-metamorphic stage (Mondou and Kaltenbach, 1979; Bray and Sicard, 1982; Galton, 1992), which is a necessity for complete metamorphosis (Gudernatsch, 1912). The cascade of gene expression (Shi, 1994) during metamorphosis is regulated by thyroid hormone and decreased levels potentially will prolong or stop metamorphosis, thereby increase mortality.

Our objectives were to study the possible effects of oral exposure to PCBs and their use as a model compound for PHAHs during tadpole development and metamorphosis. Tadpoles of the South African clawed frog (*Xenopus laevis*) and the European common frog (*Rana temporaria*) were either dosed with the technical PCB mixture Clophen A50, the single non-ortho-3,3′,4,4′,5-CB congener (PCB 126) or 2-mercapto-1-methyl-imidazole (MMI), a known goitrogen. Exposure was for a 10-day period or throughout larval development until completed metamorphosis, dosing was initiated before the thyroid hormone dependent developmental stages.

## 2. Animals, materials and methods

### 2.1. Chemicals

The crystalline PCBs (IUPAC NR. 28, 52, 53, 101, 103, 118, 126, 138, 153 and 180; all 99% purity) used for chemical analysis of tissue concentrations, and PCB 126 and the technical mixture Clophen A50, which were used for exposure of animals, were obtained from Promochem, Wesel, Germany. MMI (Janssen Chimica, Tilburg, The Netherlands), and the fixative NoTox (Earth Safe Industries, Hertogenbosch, The Netherlands) were the only chemicals used throughout the experiments not obtained from Merck, Darmstadt, Germany. $T_4$ Amerlite kits were obtained from Amersham, Aylesbury, UK.

### 2.2. Animals, breeding and housing

Adult African clawed frogs (*X. laevis*) were obtained from the Department for Experimental Zoology, Catholic University of Nijmegen, and were maintained in aquaria with constantly filtered and aerated water at 20°C at a 12:12 photoperiod. Animals were fed with beef heart or commercial trout food (Provimi Agra, Zwolle, The Netherlands) twice weekly. Viable gametes were obtained as previously described (Gutleb et al., 1999). Tadpoles of eight pairs were pooled for the experiments.

Common frogs (*R. temporaria*; six adult pairs) were collected on 30 March 1995 near Maria Saal, Carinthia, Austria (46°38′30″ N, 14°22′30″ E) before they entered a pond for breeding. Males and females were transported separately immediately after collection in 10-l boxes on ice-water covered with foam rubber to the Netherlands. On arrival 12 h later they were in good health and were further kept at $17 \pm 1$°C throughout all experiments.

The developmental stage of tadpoles were scored according to Nieuwkoop and Faber (1975) and Gosner (1960) for *X. laevis* and *R. temporaria*, respectively. Tadpoles of both species were fed a diet consisting of 500 g dried nettle powder (Jacob Hooy, Limmen, The Netherlands), 5 g coffee-creamer (Friesland Dairy Foods, Leeuwarden, The Netherlands), 5 g agar granulated and 5 g yeast extract (both from Becton Dickinson, Cockeysville, USA). The powder was mixed with tempered water (approx. 35°C) to obtain a viscous mixture, which was added once per day to the aquaria and made the water a dark green opaque colour. Water from all exposure experiments was charcoal-filtered prior to replacement.

### 2.3. Short-term feeding experiment (10-day exposure)

*Xenopus laevis* tadpoles ($n = 60$ for vehicle control and positive control groups and $n = 45$ for PCB-dosed groups) at stage 49, a stage considerably before the thyroid peak, were kept in aquaria of $29 \times 29 \times 48$ cm (total volume = 25 l, water temperature = 24°C) and were fed for 10 days a diet containing 2 or 200 mg/kg Clophen A50 or 0.2 mg/kg PCB 126. A diet with 0.2 mg/kg MMI was used as a positive control. After exposure, all animals were fed an uncontaminated diet for a period of 46 days when 75% of the tadpoles of the control group had metamorphosed (stage 65/66). PCBs and MMI were dissolved in acetone and the solutions were added to the nettle powder mix, after which acetone was evaporated in a fume-hood at room temperature for 24 h. The control group received an uncontaminated acetone treated diet. Aquaria were checked daily for dead animals. Froglets were anaesthetised in ice-water and thereafter sacrificed by cervical transection, weighed and scored for malformations. Lower jaw tissues were removed and stored at −80°C prior to analysis of $T_4$.

### 2.4. Long-term feeding experiment (exposure until end of metamorphosis)

Tadpoles of *X. laevis* (stage 50/51, $n = 120$), respectively, tadpoles of the corresponding stage of *R. temporaria* (stage 25, $n = 30$) were exposed to the same diets as in the short-term feeding experiment. The experiment was terminated after 76 days for *X. laevis* and after 51 days for *R. temporaria*, when at least 75% of the control animals were metamorphosed. *Xenopus laevis* tadpoles were further kept and treated as described for the short-term feeding experiment. *Rana temporaria* tadpoles were kept in aquaria of $18 \times 18 \times 30$ cm (total volume = 8 l, water temperature = 18°C). *Xenopus laevis* of stages 51/52, 56/57, 61/62, 62/63, and 64/65 ($n = 12$ for stages 51/52 and 56/57, respectively, $n = 4$ for all other stages) were sacrificed periodically in each group for analysis of actual thyroid hormone levels. *Rana temporaria* were not killed before metamorphosis because of the smaller number of tadpoles available. Lower jaw tissues of dead *X. laevis* were removed and stored at −80°C prior to analysis of $T_4$. Carcasses were wrapped in aluminium foil and stored at −20°C for determination of PCB concentrations.

### 2.5. Thyroid hormone assays

$T_4$ levels in the lower jaws of five tadpoles were determined following the method of Bray and Sicard (1982) with a few modifications. The protocol of the supplier (Amersham, UK) was modified by diluting the $T_4$ assay reagent five times with demineralised water and applying a $T_4$ standard curve ranging from 0 to 30 nM $T_4$/l. Thyroid hormone levels were calculated from the luminescence data with the Securia computer program of Amersham.

### 2.6. Chemical analysis

Details of sample preparation, extraction, clean-up and gas-chromatographic conditions of PCB analysis were previously described (Gutleb and Kranz, 1998). Three carcasses of each exposure group were analysed. Recovery level of reference standard samples and internal standard was always greater than 80%, and the coefficient of variation of five replicates was $< 10\%$. Limit of detection was 0.05–0.34 ng/g lipid weight for single congeners. Total PCB concentrations were calculated by addition of the individual concen-

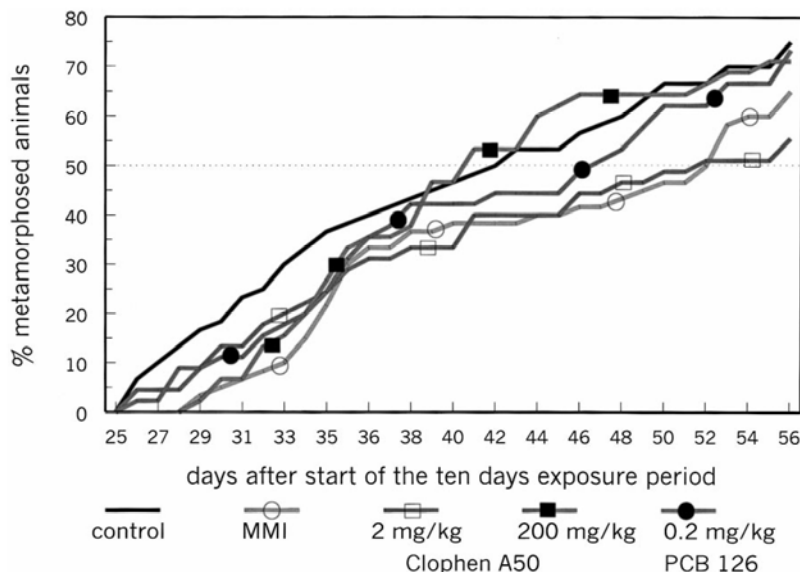


Fig. 1. Cumulative percentages of metamorphosed froglets of *Xenopus laevis* after short-term oral exposure to PCBs.

trations of seven congeners (IUPAC NR. 28, 52, 101, 118, 138, 153 and 180).

### *2.7. Statistical analyses*

All data are reported as means ± standard error of the mean (S.E.M.) with the exception of results for $n < 5$ where means ± standard deviation (S.D.) are given. Differences between treatments were tested with one-way ANOVA, respectively, Kruskal–Wallis $H$-test where appropriate. The acceptance level was set at $P < 0.05$. Statistical analyses were performed using SPSS/PC+, version 6.0 (SPSS Inc., Chicago, IL, USA).

## 3. Results

### *3.1. Short-term feeding experiment (10-day exposure) with X. laevis*

In all groups, mortality was low (2–3%) and no malformed tadpoles were observed. At the end of the experiment, 75% of the control animals had reached stage 65/66. This percentage was lower in all other groups, namely 73.3% in the group exposed to 0.2 mg/kg PCB 126, 71.1% in the group receiving 200 mg/kg Clophen A50, 65% in the MMI group, and 55.5% in the 2 mg/kg Clophen A50 group (Fig. 1).

In the groups fed a diet of 2 mg/kg Clophen A50 or 0.2 mg/kg MMI animals metamorphosed later than in the control group. In the control group and the group exposed to 200 mg/kg Clophen A50, 50% of the animals metamorphosed between 42 and 44 days after the exposure had stopped. Metamorphosis (50% of the animals) took 48 days for 0.2 mg/kg PCB 126 and 52 days for the 2 mg/kg Clophen A50 and MMI tadpoles.

Froglets of the group exposed to 2 mg/kg Clophen A50 were significantly heavier than animals from all other groups (one-way ANOVA, $P < 0.001$) (Table 1). Duration of the larval period did not differ among animals that metamorphosed ($P > 0.05$) (Table 1).

Although not significant, $T_4$ concentrations in froglets (stage 65) which received 200 mg/kg Clophen A50 and MMI were lower than in the other groups (Table 1; Kruskal–Wallis $H$-test, $P > 0.05$).

Table 1
Body weight, larval period and $T_4$ levels of metamorphosed *Xenopus laevis* from 10 days of oral exposure to PCBs — short-term feeding experiment[a]

| Exposure group | *n* | Body wt. (mg) (mean ± S.E.M.) | Duration until completed metamorphosis (days ± S.E.M.) | *n* | $T_4$ (pg/lower jaw) (mean ± S.D.[#]) |
|---|---|---|---|---|---|
| Control | 46 | 272 ± 9 | 40 ± 2 | 3 | 45 ± 29 |
| MMI | 43 | 282 ± 12 | 43 ± 2 | 3 | 25 ± 14 |
| 2 mg/kg Clophen A50 | 29 | 432 ± 30*** | 41 ± 2 | 3 | 43 ± 5 |
| 200 mg/kg Clophen A50 | 34 | 274 ± 11 | 42 ± 2 | 3 | 31 ± 7 |
| 0.2 mg/kg PCB 126 | 32 | 268 ± 12 | 41 ± 2 | 3 | 41 ± 12 |

[a] Statistical evaluation (body weight, larval period) was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Student–Newman–Keuls test that controlled Type I experiment-wise error, *** $P < 0.001$;
[#] Statistical evaluation for $T_4$ levels was made by Kruskal–Wallis H-Test, $P > 0.05$.

## 3.2. Long-term feeding experiment (exposure until end of metamorphosis)

### 3.2.1. Xenopus laevis

The overall mortality in the group receiving 200 mg/kg Clophen was 51 of 120 animals (42.5%), compared to 20.8% in the control animals. Higher mortality was observed in the group receiving 0.2 mg/kg PCB 126, 2 mg/kg Clophen A50 or 0.2 mg/kg MMI in which the overall mortality was 40.8%, 40.0%, and 37.5%, respectively. A steep increase in mortality was observed in the group receiving 200 mg/kg Clophen A50 (Fig. 2) with 43 animals dying within 9 days (day 13–22). A more gradual increase of mortality occurred in the other groups.

The percentage of dead animals with malformations was highest in the group exposed to 0.2

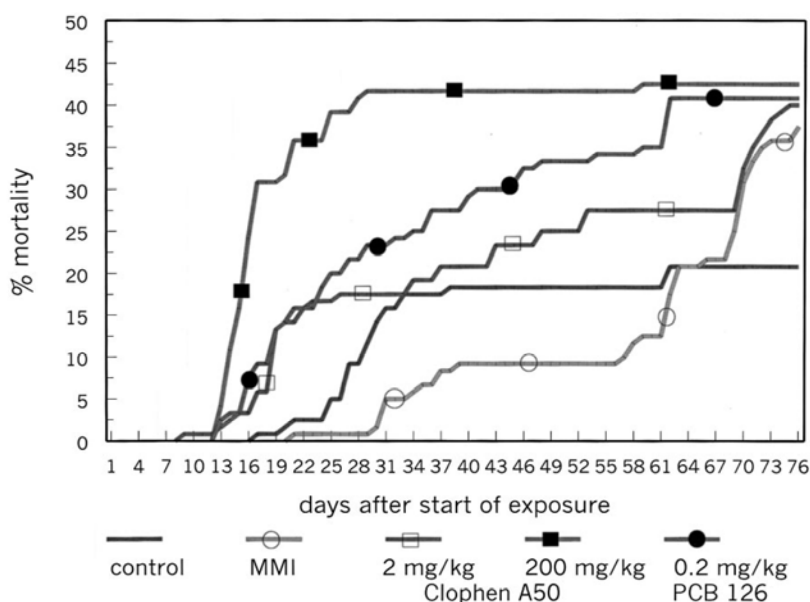


Fig. 2. Percent mortality of *Xenopus laevis* tadpoles after long-term oral exposition.

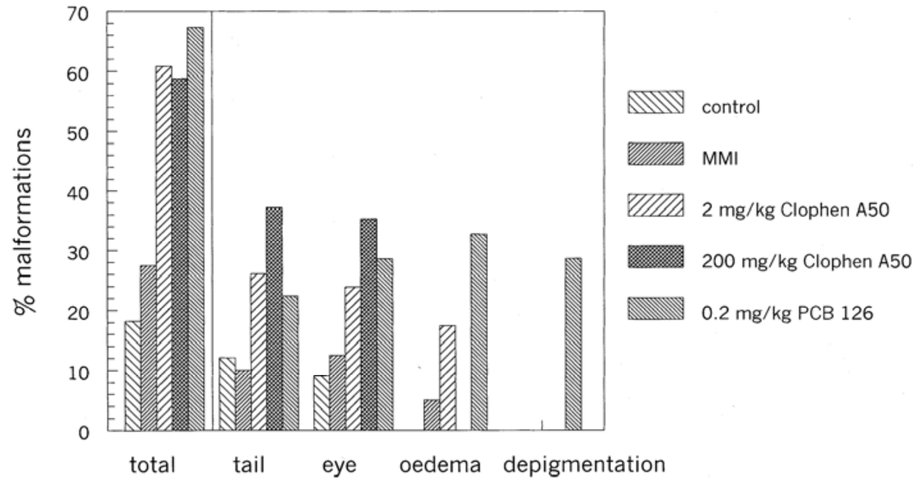


Fig. 3. Type of malformations in *Xenopus laevis* tadpoles after long-term oral exposure.

mg/kg PCB 126 (67.3%). Lower percentages of malformations were observed in tadpoles exposed to 2 mg/kg Clophen A50 (60.8%), 200 mg/kg Clophen A50 (58.8%), or 0.2 mg/kg MMI (27.5%) (Fig. 3). In the control group, 18.2% of dead tadpoles were malformed. Tail and eye deformities were the most prominent malformations in the PCB-exposed groups. Oedema was found with a high incidence only in the groups exposed to 2 mg/kg Clophen A50 and 0.2 mg/kg PCB 126. Depigmentation of the skin was observed only in the PCB 126 group.

Froglets that received 200 mg/kg Clophen A50 or 0.2 mg/kg PCB 126 in their diet were significantly heavier than animals exposed to 2 mg/kg Clophen A50 ($P < 0.05$). They were also heavier than animals from the other two groups although not significant ($P > 0.05$) (Table 2).

Time to complete metamorphosis was significantly increased (Table 2) in the groups exposed orally to 2 mg/kg Clophen A50 and 0.2 mg/kg PCB 126 as compared with the three other groups ($P < 0.01$).

$T_4$ levels in all groups were low at stage 51/52 and 56/57, followed by a steep increase to a peak (up to 60-fold higher) in stages 61/62 and 62/63,

Table 2
Body weight and length of larval period to metamorphosis in *Xenopus laevis* orally exposed to PCBs — long-term feeding experiment[a]

| Exposure group | *n* | Body wt. (mg) (mean ± S.E.M.) | Larval period (days) (mean ± S.E.M.) |
|---|---|---|---|
| Control | 10 | 191 ± 16 | 64 ± 8 |
| MMI | 8 | 194 ± 17 | 66 ± 8 |
| 2 mg/kg Clophen A50 | 5 | 155 ± 18 | 95 ± 3**[b] |
| 200 mg/kg Clophen A50 | 14 | 232 ± 10*[a] | 64 ± 2 |
| 0.2 mg/kg PCB 126 | 10 | 229 ± 20*[a] | 101 ± 3**[b] |

[a]Statistical evaluation was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Student–Newman–Keuls test that controlled Type I experiment-wise error: *[a]significant difference ($P < 0.05$) from the 2 mg/kg Clophen A50 group; **[b]significant difference ($P < 0.01$) from the three other groups.

Table 3
$T_4$ levels (mean ± S.D. in pg/lower jaw) of *Xenopus laevis* at different developmental stages orally exposed to PCBs — long-term feeding experiment

| Exposure group | 51/52 | 56/57 | 61/62 | 62/63 | 64/65 |
|---|---|---|---|---|---|
| Control | 2.9 ± 0.4 | 3.7 ± 0.7 | 130.1 ± 62.3 | 64.7 ± 19.6 | 18.1 ± 0.7 |
| MMI | 2.4 ± 0.5 | 3.1 ± 0.4 | 85.2 ± 22.6 | 110.4 ± 31.9 | 34.0 ± 39.9 |
| 2 mg/kg Clophen A50 | 3.1 ± 0.1 | 4.7 ± 0.5 | 90.7 ± 44.2 | 101.7 ± 52.0 | 59.1 ± 39.9 |
| 200 mg/kg Clophen A50 | 2.6 ± 0.2 | 5.5 ± 2.3 | 51.8 ± 15.1 | 84.4 ± 42.4 | 15.1 ± 7.1 |
| 0.2 mg/kg PCB 126 | 2.1 ± 1.4 | 5.6 ± 1.9 | 120.6 ± 10.6 | 111.7 ± 18.8 | 39.0 ± 16.5 |

after which levels decreased in metamorphosed froglets (stage 64/65) (Table 3). The standard deviation was quite high (up to 50 %) due to the rapid increase of $T_4$ and the correlated fast development of tadpoles of stage 61/62–65, with animals finishing metamorphosis within a few days after stage 61/62 has been reached. Due to the high standard deviation, $T_4$ levels were not significantly different between any of the groups.

Concentrations of PCBs in froglets from the control group and the group exposed to MMI were lower than the detection limit, but after 76 days, significant amounts of PCBs accumulated in the experimentals (Table 4). Total body burden in animals (three animals were pooled, $n = 3$) exposed to a diet of 2 mg/kg Clophen A50 was $12.5 \pm 7.1$ μg/g (lipid weight) at the end of the experiment. Concentrations in pooled samples of the group exposed to 200 mg/kg Clophen A50 were $348.1 \pm 249$ μg/g (lipid weight) and PCB 126 accumulated up to $4.8 \pm 1.5$ μg/g (lipid weight).

### 3.2.2. *Rana temporaria*

The pattern of mortality in the long-term feeding experiment (Fig. 4) was similar to that observed in the parallel experiment with *X. laevis* (Fig. 2). An overall mortality of 46.7% in the group exposed to 200 mg/kg Clophen A50 and lower mortality in the two groups exposed to 0.2 mg/kg PCB 126 (20%), and 2 mg/kg Clophen A50 (33.3%) was found. No dead animals were observed later than day 35 of exposure in any dosed group. No mortality occurred in the control and the MMI group.

In contrast to *X. laevis* no malformations were found among tadpoles, which died throughout the experiment.

Animals exposed to 0.2 mg/kg MMI were significantly lighter ($P < 0.001$), and the animals exposed to 2 mg/kg Clophen A50 were significantly heavier ($P < 0.001$) than animals from the other groups.

*Rana temporaria* exposed to MMI and to 200 mg/kg Clophen A50 needed a significantly longer period until metamorphosis (Table 5) than animals from the other groups (one-way ANOVA, $P < 0.05$). Length of larval period in the groups exposed to 2 mg/kg Clophen A50, 0.2 mg/kg PCB 126 and the control group were not significantly different from each other (one-way ANOVA, $P > 0.05$).

PCBs were not detected in the control and the MMI group, but in froglets at day 51 of exposure to 2 mg/kg Clophen A50 and 200 mg/kg Clophen A50 a total body burden of $12.0 \pm 9.8$ μg/g (lipid weight) and $559.7 \pm 229$ μg/g (lipid weight), re-

Table 4
Body burdens (mean; min–max) of metamorphosed *Xenopus laevis* and *Rana temporaria* after long-term oral exposure to PCBs (μg/g lipid weight)

| Exposure group | $n$ | *X. laevis* | *R. temporaria* |
|---|---|---|---|
| Control | 3 | n.d. | n.d. |
| MMI | 3 | n.d. | n.d. |
| 2 mg/kg Clophen A50[a] | 3 | 12.5<br>4.3–16.6 | 12.0<br>3.5–22.7 |
| 200 mg/kg Clophen A50[a] | 3 | 348.1<br>154.9–629.1 | 559.8<br>296.1–674.4 |
| 0.2 mg/kg PCB 126 | 3 | 4.8<br>3.1–5.9 | 5.4<br>3.5–7.2 |

[a] Sum of seven congeners; n.d. = not detected.

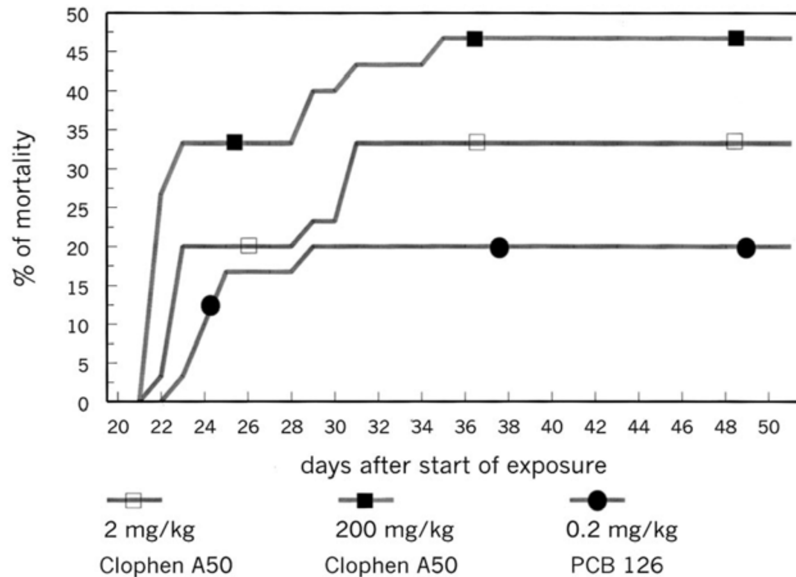


Fig. 4. Percent mortality of *Rana temporaria* tadpoles after long-term oral exposition. Note that neither in the control nor in the MMI group was mortality observed.

spectively, were detected. PCB 126 accumulated to a body burden of 5.4 ± 2.6 μg/g (lipid weight).

## 4. Discussion

Mortality in both species was observed in the long-term oral exposure experiments. Although several developmental malformations were observed (i.e. tail, eye, oedema) with *X. laevis*, a dose–response was observed only with tail and eye anomalies. In addition, depigmentation in *X. laevis* after long-term oral exposure to PCB 126 was observed. Body weight increased and duration of larval period was prolonged as a result of PCB exposure in both species. $T_4$ levels were lowered, although not significantly.

Table 5
Body weight and length of larval period to metamorphosis in *Rana temporaria* orally exposed to PCBs — long-term feeding experiment[a]

| Exposure group | *n* | Body wt. (mg) (mean ± S.E.M.) | Larval period (days) (mean ± S.E.M.) |
|---|---|---|---|
| Control | 30 | 203 ± 7 | 35 ± 4 |
| MMI | 28 | 143 ± 5*[a] | 41 ± 4* |
| 2 mg/kg Clophen A50 | 20 | 279 ± 8*[a] | 35 ± 3 |
| 200 mg/kg Clophen A50 | 16 | 177 ± 8 | 38 ± 4* |
| 0.2 mg/kg PCB 126 | 24 | 209 ± 9 | 35 ± 3 |

[a]Statistical evaluation was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Student–Newman–Keuls test that controlled Type I experiment-wise error, *$P < 0.05$; [a]significant difference from all other groups.

Table 6
Effects of PCBs on offspring of *X. laevis* and *R. temporaria* as a result of different exposure routes[a]

| Effect | Route of exposure | |
|---|---|---|
| | Oral (short) | Oral (long) |
| Mortality | n.e./n.a. | ▲/⇑ |
| Malformation | n.e./n.a. | ▲/n.e. |
| Body weight | ▲/n.a. | ↓▲/◩⬔ |
| Larval period | n.e./n.a. | ▲/⇑ |
| $T_4$ | ↓/n.a. | ↓/n.a. |

[a]*Notes:* ▲▼, *Xenopus laevis* significant increase and decrease, respectively, with PCB concentration; ↓↑, *Xenopus laevis* trends; ⇑⇓, *Rana temporaria* significant increase and decrease, respectively, with PCB concentration; ◩⬔, *Rana temporaria* trends; n.e., no effect; n.a., not analysed.

The current study was undertaken to evaluate possible effects of PCBs on amphibian metamorphic transformation through disruption of thyroid hormone dependent processes. The observed disturbance of the thyroid hormone dependent metamorphosis in conjunction with effects on body weight as a result of PCB exposure in amphibians (Table 6) may result in alteration of normal amphibian development and population recruitment.

### 4.1. Effect on mortality

Tadpoles of both species showed a dose-dependent increase in mortality as a result of long-term oral exposure to PCB spiked diets (Figs. 2 and 4), whereas in *X. laevis* no effect was found after 10 days oral exposure of stage 49 tadpoles (Table 6). Tadpoles in stages before the thyroid hormone dependent peak of metamorphosis, may be less susceptible to PCBs, than when exposed later in the thyroid hormone dependent stages of metamorphosis. Metamorphosis in anuran tadpoles involves a period of larval mouth-part degeneration and development of the adult jaws, during which the animals are unable to feed. During this developmental stage without food intake and with transformation of large parts of the tadpoles' tissues, body fat will be mobilised, thereby releasing lipophilic compounds, which will exert their toxic effects.

### 4.2. Effect on body weight, duration of larval period and thyroid hormone levels

Body weight was altered and the duration of larval period was increased in both species (Tables 1, 2, 4 and 6). The results may actually reflect the summation of two processes related with PCB toxicity. Mortality within a group will potentially increase the body weight of tadpoles due to the lowered population density within the remaining group (Richards, 1958). Lowered thyroid levels will increase the time-span until successful metamorphic transformation thereby also increasing the body weight (Fox and Turner, 1967).

Froglets in the long-term feeding experiment were lighter and the percentage of metamorphosed animals was lower over the experimental period, when compared with the control group of the short-term feeding experiment. Although housing conditions were identical in both experiments the density of animals was 2–3 times higher in the long-term feeding experiment. As the growth rate of amphibian larvae is population density-dependent, differences in larval period or mass at metamorphosis may be due to different densities. Therefore the results of the two experiments with *X. laevis* are not directly comparable.

Although $T_4$ levels were not significantly decreased due to the individual variability observed, interference of PCBs with *X. laevis* tadpole thyroid hormone homeostasis occurred to some extent in both feeding experiments. The curve of $T_4$ levels was flattened and the peak was shifted to later developmental stages after long-term oral exposure. Up to now the mechanisms of PCB-induced $T_4$ alteration in warm-blooded vertebrates, namely disturbances of the synthesis or withdrawal of the hormone from the circulation by degradation and excretion in conjunction with altered transport mechanisms (Brouwer et al., 1998) have not been investigated in amphibian larvae. As noted in other studies (Fox and Turner, 1967; Marco and Blaustein, 1998) we observed arrested metamorphosis and an increase in bodyweight in the tadpoles from exposure to thyroid hormone depleting substances.

The overall effect of prolonged duration of amphibian metamorphosis in conjunction with weight alterations on wild-living amphibians cannot be estimated yet. Several publications reported that smaller tadpole body size is correlated with lowered swimming speed and increased predation risk (Travis, 1983; Richards and Bull, 1990). On the other hand, metamorphosing at a larger body size can increase survival of terrestrial juveniles and size at first reproduction, and thereby, increase individual and, in some cases, population fecundity (Berven and Gill, 1983; Semlitsch et al., 1988; Scott, 1994).

### *4.3. Malformations*

A dose-dependent increase in tail and eye deformities was found in long-term PCB-exposed tadpoles of *X. laevis*. The observed pattern of malformations corresponds with results of studies on TCDD toxicity during early embryo development in amphibians (Birge et al., 1978; Gutleb et al., 1999) and fish (Elonen et al., 1998). Exposure of *X. laevis* embryos to PCB 126 in a prolonged-FETAX assay results in a very similar pattern of malformation later in the development (Gutleb et al., 1999). In carp (*Cyprinus carpio*), PCB 126 induces an increase of adrenocorticotropic hormone (ACTH) and cortisol, which coincides with development of oedema, thus giving evidence for possible effects of PCBs on the water balance (Stouthart et al., 1998). In contrast to *X. laevis*, no malformations were observed in *R. temporaria* tadpoles, which were exposed to the same diet. *Rana catesbeiana* tadpoles show no effects after intraperitoneal administration of 1 mg/kg 2,3,7,8-TCDD body weight (Beatty and Holscher, 1976). This gives some indication that tadpoles of the family Ranidae may be less susceptible to PHAH mediated malformations than *X. laevis*.

### *4.4. PCB concentrations in experimental animals*

The concentrations of PCBs accumulated to high concentrations in all exposed groups. There is a paucity of published literature on tissue concentrations of PCBs in amphibians. In adult *R. temporaria* from an unpolluted area in the Netherlands, levels of 0.4–0.8 μg/g lipid weight have been found (Leonards et al., 1998).

In spite of a greater exposure period, *R. temporaria* often accumulated more PCBs than *X. laevis*. Although the exact reasons for these differences are not known, it may be due to differential rates of food intake, absorption rates in the gut or general metabolism of the two species.

## 5. Conclusions

The results of tests with species from two amphibian families (Pipidae, Ranidae) demonstrate that ingested PCBs alter normal amphibian development in a time- and dose-dependent manner, similar to our results for pre- and early-life time exposure (Gutleb et al., 1999). PCB exposures resulted in increased mortality, rate of malformations, slowed growth and development of tadpoles after 51–76 days of oral exposure. Although mortality and deformity rate are notable effects, the indicated impacts on development and growth may have an equal significance on overall population fitness. *Xenopus laevis* may be a useful experimental animal for testing substances on their ability to interfere with amphibian metamorphosis. However, the use of native species may be more appropriate when attempting to determine the impact a contaminant may have in a natural setting.

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# 5

# A Synchronized Amphibian Metamorphosis Assay as an improved tool to detect thyroid hormone disturbance by endocrine disruptors and apolar sediment extracts

[*submitted*]

*Xenopus laevis* tadpoles (NF stage 50 – NF stage 57)

# CHAPTER 5

## A Synchronized Amphibian Metamorphosis Assay as an improved tool to detect thyroid hormone disturbance by endocrine disruptors and apolar sediment extracts

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H.J.H. van den Berg, Albertinka J. Murk

**Abstract**

Amphibian Metamorphosis Assays are used to evaluate potential effects of endocrine disrupting compounds on the thyroid hormone axis. In this study *Xenopus laevis* tadpoles are kept in a solution of 0.2% thiourea (TU) to arrest and correspondingly synchronise them in their development.

The advantage of this Synchronized Amphibian Metamorphosis Assays is that synchronised tadpoles are available at any time to start metamorphosis experiments, and experimental groups are much more homogenous at the start of experimental exposure compared with groups selected from an untreated pool of animals. The water volume per animal was kept constant throughout the experimental period to overcome the influence of declining numbers of animals per aquarium due to metamorphosis and mortality on the density dependent development of the remaining tadpoles. Thyroid hormone disrupting compounds, namely Clophen A50 (a technical PCB mixture), the single congener 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) and apolar sediment extracts that were previously tested positive in the T-Screen, an *in vitro* proliferation assay for thyroid hormone disruption, were tested in the Synchronized Amphibian Metamorphosis Assay. Endpoints studied were mortality, malformations, body weight, and percentage of metamorphosed froglets at the end of the 60-day experimental period, percentage of tadpoles in different developmental stages, and developmental stage-dependent awarded penalty points. Dietary exposure to Clophen A50 (0.2 - 50 mg/kg food) resulted in a significant increased percentage of tadpoles that did not pass metamorphosis at concentrations higher than 2 mg/kg food. Time until metamorphosis in those animals that were able to metamorphose after the 60 days experimental period was significantly decreased. Dietary exposure to PCB 77, a congener that can be readily metabolised, did not result in significant effects in any exposure group (2 - 500 µg/kg food). Apolar sediment extracts from two of the three sites that are contaminated with a wide variety of chemicals significantly decreased the percentage of metamorphosed animals and significantly increased the number of tadpoles that remained in early and late metamorphic stages. These effects already occurred when the extracts where diluted more than 1000 times (on an organic carbon base) compared to environmental concentrations. The rank of potency was comparable to results obtained with the T-screen. This suggests the presence of thyroid hormone disrupting compounds in the aquatic environment and possible effects of such compounds on animal development in the wild.

Keywords – in vivo functional assay, dietary exposure, PCBs, thiourea, Xenopus, tadpole

# INTRODUCTION

The presence of chemicals in the environment with the potential to disrupt endocrine systems, endocrine disrupting compounds (EDCs), has become a major focus of research during the last years, as both wildlife and humans may be affected (Tyler et al., 1998; Vos et al., 2000). Whereas a lot of studies deal with (anti)estrogenic effects and related test systems (Sumpter, 1998; Legler et al., 2000), far less effort has been put in the identification of thyroid hormone disrupting compounds (Brouwer et al., 1998). It has been suggested that thyroid hormone disrupting compounds may contribute to the observed global decline of amphibian species (Blaustein, 1994; Pechman and Wilbur, 1994).

Thyroid hormones, 3,3′5-triiodo-L-thyronine ($T_3$) and 3,3′,5,5′-tetraiodo-L-thyroxine ($T_4$), have a wide range of biological effects in vertebrates both in fetal and prenatal development (Porterfield and Hendrich, 1993; Tata, 1999; Power et al., 2001). Thyroid hormones play an important role in many vertebrate classes such as in the development of sexual organs and the central nervous system in mammals (Bernal and Nunez, 1995), or in the metamorphosis of amphibians (Gudernatsch, 1912; Kanamori and Brown, 1996). Therefore the necessity to pay more attention to compounds that possibly interfere with this hormone system has been clearly formulated (EDSTAC, 1998; DeVito et al., 1999; Colborn, 2002). A number of *in vitro* tests already exist such as the T-Screen (Gutleb et al., 2005), thyroid peroxidase assay (DeVito et al., 1999; Baker, 2001), deiodinase assays (DeVito et al., 1999; Baker, 2001), and $T_4$-TTR-competition binding studies (Lans et al., 1993; Janosek et al., 2006). However these assays only focus on single aspects of thyroid hormone dependent metamorphosis and like all *in vitro* assays does not take complex feedback mechanisms and toxicokinetics into account. Therefore there is a need for functional

*in vivo* screening assays for thyroid active compounds (Brouwer et al., 1998; Fort et al., 1999, 2000; Herkovits et al., 2002).

A process where thyroid hormone plays a very specific and crucial role is completion of amphibian metamorphosis (Bray and Sicard, 1982; Galton, 1992). The first stage of tadpole development is characterized by development of larval structures and body growth (early metamorphosis, up to NF stage 55 (Nieuwkoop and Faber, 1975, further referred to as NF), followed by further growth and development of extremities (metamorphosis, up to NF stage 60). This metamorphic development up to NF stage 60 is dependent on a surge of thyroid hormone. The following stages up to completion of metamorphosis are characterised by remodelling of the body shape such as completion of extremities, tail resorption and decreasing thyroid hormone levels (late metamorphosis, up to NF stage 66) (Etkin, 1932; Nieuwkoop and Faber, 1975).

Test systems based on thyroid hormone dependent processes using tadpoles have been suggested as useful tools to study the thyroid hormone disrupting effects of single compounds, mixtures or environmental extracts (EDSTAC, 1998; Opitz et al., 2005). Examples are 30-day limb development, 14-day tail resorption studies or a 28-day Xenopus Metamorphosis Assay (Fort et al., 1999, 2000; Christensen et al., 2005; Opitz et al., 2005).

However, until now, *in vivo* assays with tadpoles require a relative large number of animals (Gutleb et al., 2000; Goleman et al., 2002). Furthermore, the rate of tadpole development differs greatly, even between individuals from the same clutch. Also the most careful selection procedure cannot completely overcome the problem that slight differences in individual development at the start of such experiments will introduce a large variation in the final outcome. To overcome this intrinsic problem of introducing variation during the process of animal selection and to make the planning of experiments more convenient by having a constant pool of animals in the right

developmental stage, we designed the Synchronized Amphibian Metamorphosis Assay. For the Synchronized Amphibian Metamorphosis Assay early metamorphic *Xenopus laevis* tadpoles are reversibly arrested in their development in NF stage 54. This is done by inhibiting type 1 deiodinase by exposure to 0.2% thiourea (TU) in NF stage 50-51 (Gorbman and Evans, 1943; Gordon et al., 1943, 1945) (see also page 20 of this thesis). Animals can be kept in this stage until they are required for an experiment. Exposure to the compounds starts 7 days before allowing them to develop further by removing the TU Functional endpoints of the Synchronized Amphibian Metamorphosis Assay are mortality, rate of malformations, body weight, duration and percentage of completed metamorphosis at the end of the 60-day experimental period, percentage of tadpoles in different developmental stages at the end of the experimental period, and developmental stage dependent awarded penalty points. As tadpole growth is density dependent (Werner, 1986; Scott, 1994) it is required that the water volume is corrected for the number of remaining tadpoles per experimental group when tadpoles are removed because they completed metamorphosis or died. The thus newly developed protocol for the Synchronized Amphibians Metamorphosis Assay was applied to test the effects of the known thyroid hormone disrupting compounds Clophen A50 (a technical PCB mixture), 3,3',4,4'-tetrachlorobiphenyl (PCB 77), and apolar sediment extracts, that were tested earlier in the T-Screen (Gutleb et al., 2005). Dietary exposure was chosen, as this is more realistic for the field situation for many lipophilic compounds with low concentrations in the water phase and higher concentrations in feed (Patyna et al., 1999).

## Animals, Materials and Methods

*Chemicals*

The technical PCB mixture Clophen A50 was a kind gift of Jan Boon (NIOZ, Den Burg, The Netherlands). PCB 77 (99% purity) was obtained from Promochem (Wesel, Germany). All other chemicals used throughout the experiments were of analytical grade and were obtained from Merck (Darmstadt, Germany).

*Animals, breeding and housing*

All experiments were performed at the Toxicology Section, Wageningen University. Adult African clawed frogs (*X. laevis*) were obtained from the Department for Animal Ecology and Ecophysiology, Radboud University of Nijmegen). Housing of adult animals and induction of egg-laying were performed as described earlier (Gutleb et al., 2000).

Tadpoles were fed a diet consisting of 500 g dried nettle powder (Jacob Hooy, Limmen, The Netherlands), 5 g coffee-creamer (Friesland Dairy Foods, Leeuwarden, The Netherlands), 5 g agar granulated and 5 g yeast extract (both from Becton Dickinson, Cockeysville, USA). The powder was mixed with tap water to get a viscous mixture, which was added once a day to the aquaria to achieve a dark green opaque colour. Feeding of larvae started on day 4 post-fertilization, and tadpoles were allowed to undergo normal development until NF stage 50-51. The developmental stage of the tadpoles was scored according to the scheme of Nieuwkoop and Faber (1975). Animals that reached NF stage 50-51 were placed in water containing 0.2% (w/v) thiourea (TU), which was completely replaced once a week.

Stocks of the exposure chemicals were dissolved to 1 mM in acetone and adequate amounts were added to 20 g nettle powder that was

already premixed with acetone to a viscous mixture. Thereafter the mixture was thoroughly stirred manually for 5 minutes, after which acetone was evaporated completely in a fume-hood at room temperature overnight. The control group received an uncontaminated acetone treated diet. Water from all exposure experiments was charcoal-filtered prior to disposal. Room temperature in experiment 1 was set to 21 °C whereas in experiment 2 the temperature was set to 24 °C throughout the complete experimental periods to increase the developmental speed of the animals.

*Preparation of sediment extracts*

Sediment samples were collected from three known polluted sites in the Netherlands, namely the North Sea Channel close to IJmuiden, the Westerschelde close to Terneuzen, and the Dommel close to Eindhoven (Vethaak et al., 2002). Portions of sediment (10 x 2g) were vortexed (1 min) after addition of 2 ml of acetone and allowed to stand for 10 minutes prior to vortexing (1 min) again. A mixture (4 ml) of hexane/dieethylether (Hx-DEE 9:1) was added and after vortexing (1 min) samples were centrifuged for 2 minutes (400g) and the upper layer of all ten parallel extracts was transferred into pre-washed glass tubes. This step was performed 3 times after which 200 µl Hx-DEE was added and the upper layer was collected again. Solvent was evaporated at 30 °C under a gentle stream of nitrogen. Before complete evaporation the organic phases of the sample were quantitatively pooled in a single vial. Hexane (500 µl) was added and the sample was filtered over a 1 g $Na_2SO_4$ column to remove non-hexane-soluble particles and possible small water remains. Before hexane was completely evaporated 20 µl DMSO was added to the extract to prepare the apolar extract stock.

*Experimental protocol*

Developmentally arrested tadpoles (NF stage 54) were randomly taken from a pool of animals with similar size and divided in groups of 10 animals. Tadpoles were kept in aquaria of 18x18x30 cm containing 400 ml of water per animal.

TU treatment and dietary exposure of compounds overlapped 7 days, after which the animals were transferred to water without TU and fed with spiked food until completion of metamorphosis (NF stage 65/66).

Tadpoles were checked daily and dead animals and/or animals that reached metamorphic NF stage 65/66 were removed. Water volume was reduced with 400 ml per removed animal. Animals reaching NF stage 65/66 were anaesthetised in ice-water, sacrificed by cervical transection and were weighed and scored for malformations. At the end of the 60-day experimental period all remaining tadpoles were anaesthetised in ice-water, sacrificed by cervical transection and their developmental stage was scored according to Nieuwkoop and Faber (1975). The results are presented in three different ways starting simply by comparing percentages of animals that passed metamorphosis as defined by reaching NF stage 65.

Secondly tadpoles are ranked into three categories according to their development, namely stages before autonomous thyroid hormone production (NF stages <55; early metamorphic), peak thyroid hormone production (NF stages 56–60, metamorphic) and late metamorphic stages (NF stages 61-65) in which TH production is decreasing (Etkin, 1932). Thirdly penalty points are accredited based on the number of developmental stages lacking until finishing metamorphosis. Animals are accredited 0 penalty points when they reached NF stage 65, 1 for NF stage 64 and so forth indicating the number developmental stages lacking for animals that have not finished metamorphosis (Gutleb et al., submitted).

*Animal exposure*

Experiment 1: Tadpoles were fed a diet spiked with Clophen A50 (0.2 mg/kg, 2 mg/kg, 10 mg/kg, 50 mg/kg) or a control diet (n=10 animals in duplicate).
Experiment 2: Tadpoles (10 animals per group) were fed a diet spiked with PCB 77 (2 µg/kg, 20 µg/kg, 100 µg/kg, 500 µg/kg) or with apolar sediment extracts (extract of 1 g sediment/kg food or of 10 g sediment/kg food) from Dommel, Terneuzen, and the North Sea Channel or a control diet. The dilution factors of 1000 and 100 times respectively were calculated on a dry weight basis, but as food consists for 100% of organic matter where sediment does not, the dilution factors are even higher on an organic matter basis.

*Data analysis*

All data in the tables are reported as means ± standard deviation. Differences between means were tested with one-way ANOVA. The acceptance level was set at $P<0.05$. Analyses were performed using SPSS/PC+, version 6.0 (SPSS Inc., Chicago, IL, USA). The scores for the animals that passed metamorphosis and the stages of tadpoles that did not pass metamorphosis were compared between treatments by $\chi^2$ contingency analysis using GraphPad Prism 4 (San Diego, CA, USA).

## RESULTS

Exposure of NF stage 50-51 tadpoles to 0.2% TU did arrest their development in NF stage 54 and the animals were kept under these conditions for up to six months until the start of an experiment without any signs of malformations or mortality.

Mortality was low (<15%) in all experimental groups and never dose related and none of the animals showed malformations. An increase in room temperature from 21 °C (experiment 1) to 24 °C (experiment 2) resulted in a higher number of metamorphosed tadpoles within the experimental period of 60 days. Body mass of metamorphosed froglets (NF stage 65) was not significantly different from control animals in any of the exposed groups either expressed as wet weight or as dry weight (one-way ANOVA, $P>0.05$).

Mean duration of larval period in Clophen A50 exposed animals that finished metamorphosis within the 60 days experimental period was shorter than in animals that finished metamorphosis in the control group (one-way ANOVA, $P<0.05$) (Table 1).

Exposure to the lowest concentration of Clophen A50 (0.2 mg/kg) resulted in a higher number (n=9) of animals that passed metamorphosis during the 60-day experimental period (Table 1). This percentage was lower in the groups exposed to 2 mg/kg Clophen A50 (n=4), 10 mg/kg Clophen A50 (n=4) or 50 mg/kg Clophen A50 (n=5) compared to the control groups (n=6). None of the tadpoles remained in early metamorphic stages (≤ NF stage 55) after 60 days in any experimental group. All animals exposed to 50 mg Clophen A50/kg feed that did not metamorphose already were in late metamorphic stages (NF stages 61-64). In the other Clophen A50 exposure groups, statistically significant more animals also were halted in development at NF stage 65-60 compared to control (Figure 1).

**Table 1.** Number of successfully metamorphosed tadpoles and duration (days; mean ± SD) until successful metamorphosis after exposure to Clophen A50 (duplicates of 10 animals), PCB 77 or apolar sediment extracts of 3 contaminated sites in the Netherlands (single group of 10 animals). The total experimental period was 60 days starting from NF stage 54.

| exposure | N (of 2 x 10) | Duration (days) |
|---|---|---|
| Experiment 1 | | |
| control | 6/6 | 49.6 ± 4.4 |
| 0.2 mg/kg Clophen A50 | 9/9 | 39.6 ± 6.2* |
| 2 mg/kg Clophen A50 | 4/5 | 41.8 ± 3.8* |
| 10 mg/kg Clophen A50 | 4/5 | 40.0 ± 5.2* |
| 50 mg/kg Clophen A50 | 4/6 | 39.4 ± 4.9* |
| Experiment 2 | N (of 10) | |
| control | 10 | 42.4 ± 7.4 |
| 2 µg/kg PCB 77 | 8 | 34.8 ± 10.9$^{(*)}$ |
| 20 µg/kg PCB 77 | 7 | 37.5 ± 13.2$^{(*)}$ |
| 100 µg/kg PCB 77 | 6 | 31.8 ± 10.3$^{(*)}$ |
| 500 µg/kg PCB 77 | 7 | 33.8 ± 14.5$^{(*)}$ |
| North Sea Channel low | 9 | 36.7 ± 11.5$^{(*)}$ |
| North Sea Channel high | 8 | 37.0 ± 12.2$^{(*)}$ |
| Terneuzen low | 6* | 38.5 ± 13.2$^{(*)}$ |
| Terneuzen high | 3* | 34.3 ± 6.5$^{(*)}$ |
| Dommel low | 5* | 50.6 ± 10.0$^{(*)}$ |
| Dommel high | 4* | 42.0 ± 12.9 |

(low = extract of 1 g sediment/kg feed; high = extract of 10 g sediment/kg feed) Statistical evaluation for the number of metamorphosed tadpoles was done by $\chi^2$ contingency analysis, *P*<0.05; Statistical evaluation for the duration of larval period was made by one-way ANOVA using Student-Newman-Keuls test that controlled Type I errors, $^{(*)}$ P<0.1; * *P*<0.05; tadpoles that did not metamorphose within 60 days are not included in the statistics

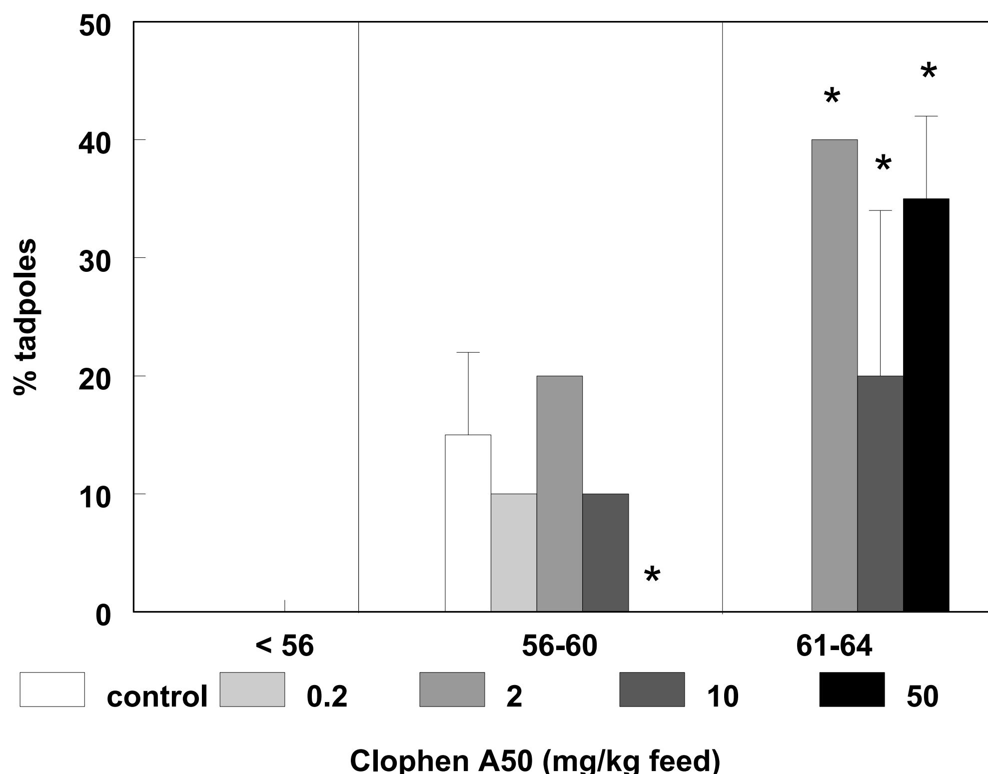


**Figure 1.** Distribution of tadpoles that did not metamorphose within 60 days of dietary exposure to Clophen A50 (mg/kg food) into three developmental categories corresponding with early, peak and late metamorphosis. Results are mean ± SD for 10 animals/group, 2 groups/concentration; Kruskal Wallis H-Test, * $P<0.05$. Results were identical for several of the duplicates, in those cases the standard deviation=0.

The mean number of developmental penalty points awarded to individual tadpoles from the exposed groups at the end of the experimental ranged from 3.3±1.2 (50 mg/kg feed Clophen A50) to 6.6 ±1.1 (control). The total number of penalty points awarded to tadpoles ranged from 6.0±1.8 (0.2 mg/kg feed Clophen A50) to 19±2.5 (2 mg/kg feed Clophen A50). Neither the mean number of penalty points nor the total number of penalty points per experimental group differed significantly for any of the groups exposed to Clophen A50 compared with the control groups (Kruskal Wallis H-Test, $P>0.05$).

In the PCB 77 exposed groups no significant effects were observed in the Synchronized Amphibian Metamorphosis Assay, only trends (one way ANOVA, $P<0.1$). Animals that did finish metamorphosis needed a shorter period (Table 1). The animals that did not finish mostly were in NF stages 61-64 at the end of the experiment in all groups (data not shown).

The number of developmental penalty points differed significantly only for the group exposed to 100 μg/kg PCB 77 compared with the control group (Kruskal Wallis H-Test, $P<0.05$) (Figure 2).

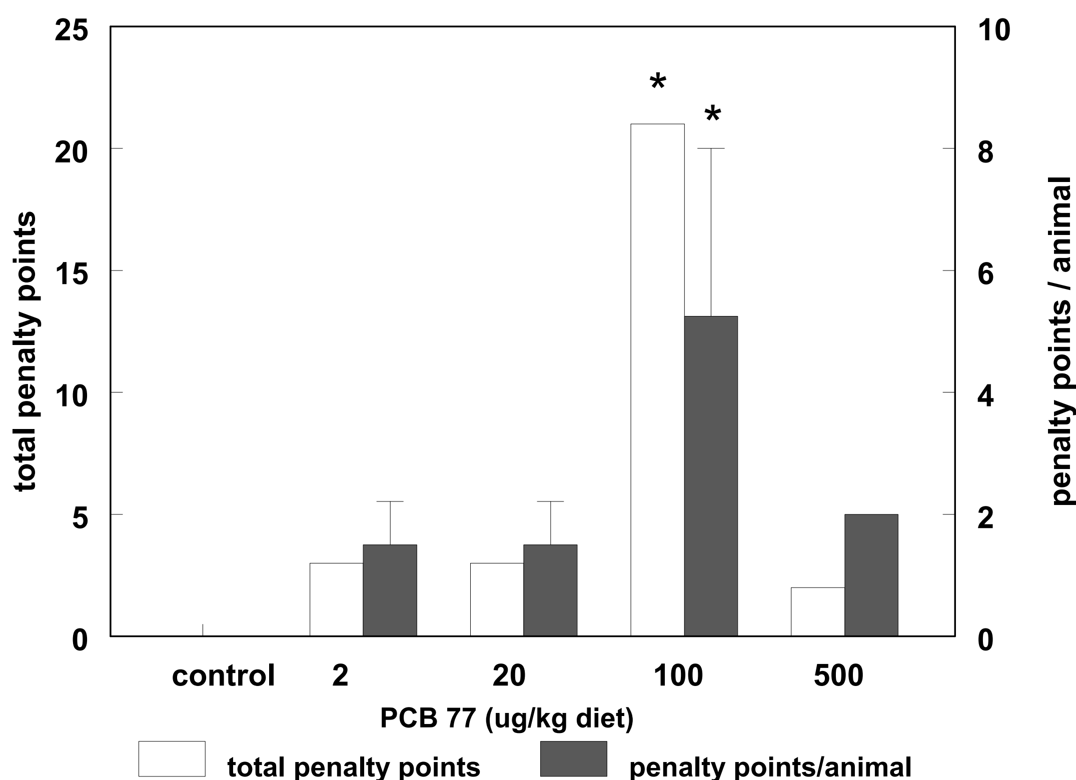


**Figure 2.** Total penalty points/group of ten animals and mean penalty points/animal (n=10) after 60 days of dietary exposure to PCB 77. Animals reaching NF stage 65 were counted as 0; NF stage 64 was 1 and so forth. Statistical evaluation was made by Kruskal Wallis H-Test, * $P<0.05$

Exposure of tadpoles to the apolar sediment extracts from Dommel and Terneuzen, but not North Sea Channel, resulted in dose dependent significantly lowered number of metamorphosed animals (Table 1). Exposure did not significantly alter duration until

metamorphosis. However a trend was seen to shorter duration until metamorphosis in animals exposed to apolar sediment extracts from the North Sea Channel and Terneuzen, and a longer period in the animals exposed to low dose Dommel extract (one-way ANOVA, $P<0.1$, Table 1).

Apolar sediment extracts from Terneuzen caused a significant delay in development with a high number of the exposed tadpoles that were either still in the metamorphic stages (NF stage 55-60) ($\chi^2=12.66$; d.f.=6; $P=0.049$) or late metamorphic stages ($\chi^2=12.06$; d.f.=6; $P=0.06$). The tadpoles exposed to the sediment extracts of Dommel were halted in the late metamorphic stages (NF 61-64) ($\chi^2=12.06$; d.f.=6; $P=0.06$). These effects on metamorphosis are either significant or a trend ($P<0.1$) and this is also reflected in the dose-related increase in the cumulative penalty points per exposure group and average penalty points per animal after exposure to apolar sediment extracts from Terneuzen and Dommel. The dose-related increases in penalty points for the animals exposed to apolar sediment extracts from the North Sea Channel were not significant due to the small number of tadpoles (n=2) in this group (Figure 3).

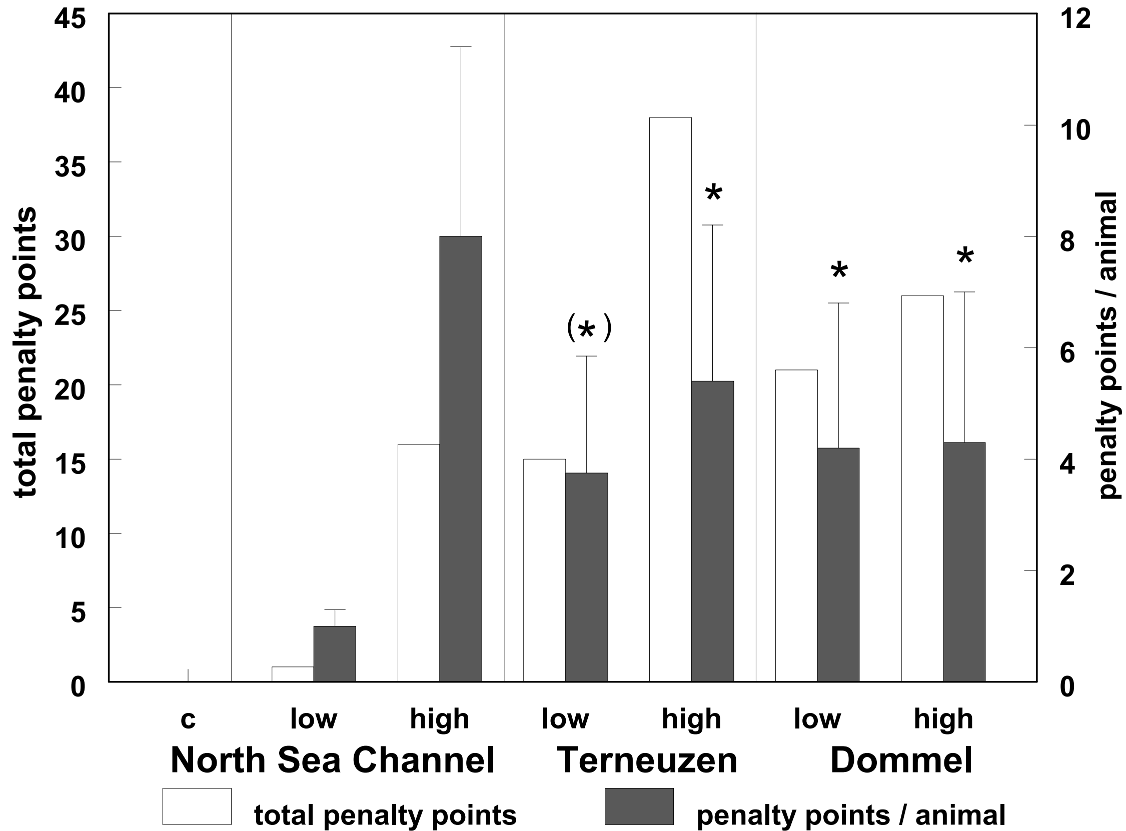


**Figure 3.** Average total penalty points/group of ten animals and mean penalty points/ animal (n=10) after 60 days of dietary exposure to apolar sediment extracts. Animals reaching NF stage 65 were counted as 0; NF stage 64 was 1 and so forth. Statistical evaluation was made by Kruskal Wallis H-Test, (*) $P$<0.1; * $P$<0.05

## Discussion

The method introduced in this paper to developmentally synchronise tadpoles with TU, resulted in a pool of animals that behaved normally (feeding, growing) relative to untreated animals without any increase in tadpole mortality or malformations. Previous research has pointed out that these animals remain thyroid hormone responsive (Rot-Nikcevik and Wassersug, 2003), and to our best knowledge there are no irreversible effects of TU on TH homeostasis known.

Animals maintained under these conditions can be kept developmentally arrested for at least six months, creating a homogenous pool of animals at the start of an experiment, thereby wasting fewer animals that are in different stages of development than in current protocols (Gutleb et al., 2000; Opitz et al., 2005).

Mortality and malformation rate was much lower than the 15% mortality considered being acceptable for the early life stage FETAX test (ASTM, 1991). This rate was also lower than in earlier classical metamorphosis experiments when younger animals (n=120) of NF stage 50/51 were used that have not been synchronised (Gutleb et al., 2000). In those tests a peak of mortality (up to 35%) was observed in the first 21 days after the start of exposure. The exposure in the Synchronized Amphibian Metamorphosis Assay starts at the later developmental NF stage 54.

The synchronisation procedure introduced here could also facilitate availability of tadpoles for the 30-day limb development or 14-day tail-resorption studies starting at NF stage 60 (Fort et al., 1999; 2000). However, the advantage of our longer exposure protocol starting with NF stage 54 is that it covers a longer part of the life cycle and the animals can be exposed a longer period and can build up a realistic body burden before the peak of thyroid hormone levels at NF stage 62 (Bray and Sicard, 1982; OECD, 2005). A longer exposure period also reduces the chance that still some thyroid hormone is stored in the thyroid producing cells possibly masking effects of the disrupting compound under investigation (Goleman et al., 2002).

Under the improved experimental conditions, with constant water volume/animal, no significant alterations of body mass were observed, whereas weight increased in earlier experiments in the

groups with high mortality due to Clophen A50 or PCB 126-exposure without adaptation of the water volume (Gutleb et al., 2000).

This is in accordance with reports in literature about the influence of animal density on both the duration of metamorphosis and the weight of froglets (Richards, 1958; Flores-Nava and Vera-Muñoz, 1999; Browne et al., 2003). The constant animal density throughout the experimental period is an important factor to be included in every metamorphosis protocol.

The Synchronized Amphibian Metamorphosis Assay aims to detect more subtle effects on the thyroid hormone dependent metamorphosis rather than general toxic effects as mortality and malformation. Dietary exposure to Clophen A50 resulted in an increased rate of metamorphosis in the lowest concentration and in a decreased rate of metamorphosis at the higher concentrations that strikingly reflect hormesis and the commonly observed maximum stimulatory response of 130-160% compared to control values (Calabrese, 2002). Such an alteration of metamorphosis was described earlier in tadpoles (Gutleb et al., 2000). Whether the increased rate of metamorphosis observed after exposure to the lower concentrations of Clophen A50 is due to a thyroid hormone-like stimulus cannot be concluded at the present. Mechanisms of interference with thyroid hormone homeostasis by Clophen A50 and other PCB mixtures include both the transport, metabolization and mimicking of thyroid hormones (Brouwer et al., 1998).

Dietary exposure to PCB 77 did not significantly influence the number of metamorphosed *Xenopus laevis* froglets, although the concentrations covered a comparable concentration range as Clophen A50 when compared on a dioxin-equivalent basis (van den Berg et al., 1998). This may be explained by the fact that PCB 77 is

readily metabolised whereas the technical mixture Clophen A50 holds several congeners that can hardly be metabolised (e.g. PCB 126). PCB 126 decreased the number of *Rana sp.* metamorphosing tadpoles exposed shortly before the TH surge after exposure via the water phase (Rosenshield et al., 1999).

Sediment extracts from the North Sea Chanel, Dommel and Terneuzen decreased the number of tadpoles that metamorphosed within the experimental period in a dose-dependent manner. These effects expressed as developmental penalty points were statistically significant for sediment extracts from Dommel and Terneuzen. This reveals that the hexane-extractable compounds were very potent as the sediment extracts applied were diluted 100 and 1000 times when expressed on a dry weight basis, but this is even more when compared with sediment levels on organic carbon basis. The potency of sediment extracts to decrease the rate of metamorphosis was Dommel > Terneuzen > North Sea Channel and this rank fits to results of the T-Screen for the identical extracts (Gutleb et al., 2005). This further suggests an effect via thyroid hormone dependent processes. A decreased percentage of metamorphosed tadpoles has earlier been reported after water-phase exposure of tadpoles to sediment extracts (Fort et al., 1999). Sediments from the same locations in the Netherlands have recently been shown to contain unidentified lipophilic compounds with thyroid-hormone mimicking ability as apolar sediment extracts have been shown to displace thyroxine ($T_4$) from transthyretin in the $T_4$-TTR assay (Houtman et al., 2004). This further suggests an effect via disruption of the thyroid hormone system as a possible explanation for our results in the Synchronised Amphibian Metamorphosis Assay. Both increased or decreased levels of $T_3$ and $T_4$ and their ratio will affect the thyroid hormone regulated timing of amphibian metamorphosis (Huang et al., 2001).

The ecological relevance of the observed alterations of tadpole development for wild-living amphibian populations, both via effects on the individual lifecycle and on the overall population recruitment, cannot yet be determined. Nevertheless the increased duration of metamorphosis relative to availability of food resources may lower the survival rate and increase the risk of predation especially during the period of metamorphic climax when the animals are sluggish (Travis, 1983; Richards and Bull, 1990; Schriks et al., in press).

## CONCLUSION

Synchronisation of amphibian metamorphosis has several advantages such as the possibility to plan availability of animals long in advance, availability of a more homogenous group of animals in identical developmental stages thus reducing the number of wasted experimental animals. Adaptation of water volume to the number of animals is important to prevent false positive effects via the indirect influence on weight and developmental rate of remaining animals. Clophen A50 was able to significantly alter thyroid hormone dependent amphibian metamorphosis whereas no such effects were observed for PCB 77 at the concentrations used. The developmental penalty points as a measure of changed speed of metamorphosis is able to combine information on reduced numbers of finished metamorphosis and severity of this reduction. The Synchronised Amphibian Metamorphosis Assay revealed that apolar sediment extracts have the potential to significantly impair amphibian development already at concentrations of more than 100 respectively 1000 times less compared to sediment concentrations. The rank of potency of the sediment extracts was identical to that obtained for effects of the sediments on thyroid hormone dependent cell growth in the T-Screen.

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# T-Screen as a tool to identify thyroid hormone receptor active compounds

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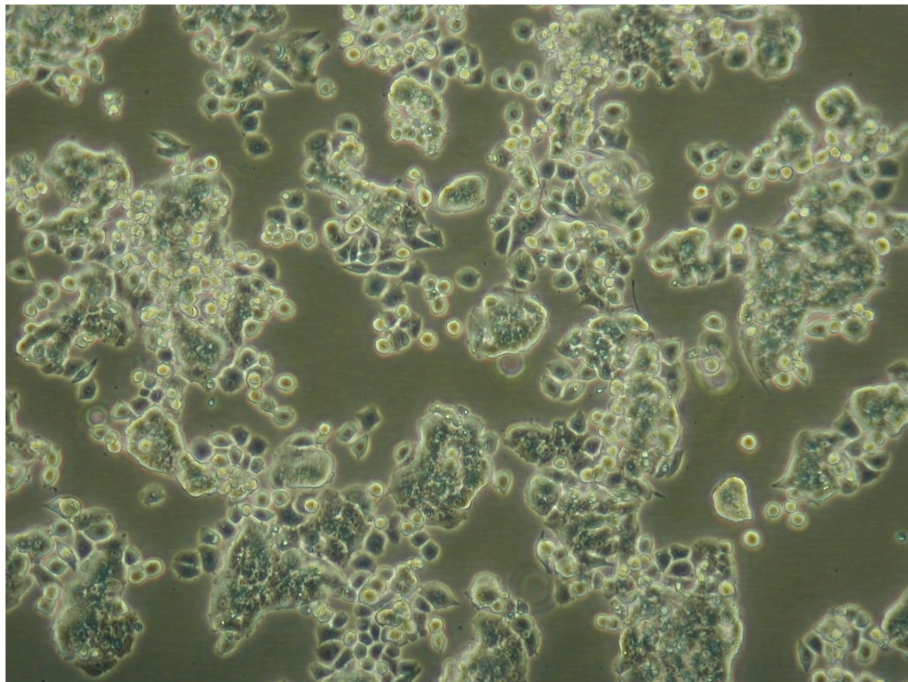

GH3 cells in culture
(Photo courtesy of Merijn Schriks)

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# T-Screen as a tool to identify thyroid hormone receptor active compounds

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**Abstract**

The T-Screen represents an in vitro bioassay based on thyroid hormone dependent cell proliferation of a rat pituitary tumour cell line (GH3) in serum-free medium. It can be used to study interference of compounds with thyroid hormone at the cellular level, thus bridging the gap between limitations of assays using either isolated molecules (enzymes, transport proteins) or complex in vivo experiments with all the complex feedback mechanisms present. Compounds are tested both in the absence and presence of thyroid hormone ($EC_{50}$ concentration of $T_3$) to test for both agonistic and antagonistic potency.

Thyroid hormones (3,3′-5-triiodothyronine: $T_3$ and 3,3′,5,5′-tetraiodothyroxine: $T_4$) and compounds resembling the structure of thyroid hormones (3,3′-5-triiodothyroacetic acid: Triac; 3,3′,5,5′-tetraiodothyroacetic acid: Tetrac) induced cell growth, with the rank order Triac > $T_3$ > Tetrac > $T_4$ (relative potency = 1.35 > 1 > 0.29 > 0.07), which is identical to published affinities of these compounds for nuclear thyroid hormone receptors. Exposure to 5,5′-diphenylhydantoin (DPH) in the presence of 0.25 nM $T_3$ resulted in up to 60% decreased cell growth at 200 μM DPH. No effect of DPH on basal metabolic activity of GH3 cells was observed at this concentration. Fentinchloride ($IC_{50}$ = 21 nM) decreased cell growth induced by 0.25 nM $T_3$, whereas parallel exposure to these concentrations in the absence of $T_3$ did not alter basal metabolic activities of GH3 cells. Apolar sediment extracts from the Dommel (34%) and Terneuzen (14%) decreased cell growth in the presence of 0.25 nM $T_3$, whereas the extract from Hoogeveen increased cell growth (26%) and the extract from North Sea Channel had no effect.

The T-Screen proved to be a fast and functional assay for assessing thyroid hormone receptor active potencies of pure chemicals or environmental mixtures.
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*Keywords:* Thyroid hormone disruptor; EDC; Bioassay; In vitro

## 1. Introduction

Thyroid hormones (3,3′-5-triiodothyronine: $T_3$ and 3,3′,5,5′-tetraiodothyroxine: $T_4$) have a wide range of biological effects in vertebrates both in foetal and prenatal development (Porterfield and Hendrich, 1993; Tata, 1999; Power et al., 2001) and have an important role in the development of sexual organs and the central nervous system in mammals (Bernal and Nunez, 1995) in the metamorphosis of amphibians (Gudernatsch, 1912), the transformation of salmon from freshwater-dwelling par to seawater-dwelling smolts (Specker, 1988) or flounder metamorphosis (Inui and Miwa, 1985).

Several classes of environmental contaminants or their metabolites can alter thyroid hormone homeostasis through interference with the thyroid hormone signal transduction pathway and associated cellular functions (Brouwer et al., 1998; Brucker-Davis, 1998; Meerts et al., 2000). These compounds or their metabolites are known to interact at the level of the thyroid hormone gland, thyroid hormone metabolism, thyroid hormone receptor or with thyroid hormone transport proteins (Brouwer, 1989; Lans et al., 1993; Murk et al., 1994a; Meerts et al., 2000). A wide variety of compounds are known

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to interfere on a cellular level with thyroid hormones (Zemel et al., 1988; Norman and Lavin, 1989; Topliss et al., 1989; Bogazzi et al., 2001; Kitamura et al., 2002). The relevance of this exposure to thyroid hormone disrupting compounds has been shown in the past for many vertebrate species both under experimental conditions and in the field (Brouwer et al., 1989; Gutleb et al., 2000; Manzon et al., 2001; Murk et al., 1994b). Subchronic exposure to an environmentally relevant mixture of 16 organochlorines at subchronic concentrations was shown to alter hypothalamic–pituitary–thyroid axis dependent endpoints in male rats (Wade et al., 2002).

For most chemicals and mixtures, but also extracts from matrices taken in the field such as sediments, the potential effects on thyroid hormone functions are still unclear. The T-Screen is a cell-based assay that was developed in order to bridge the gap between limitations of assays such as thyroid peroxidase assay, deiodinase assays and in vitro $T_4$-TTR-competition binding studies (DeVito et al., 1999; Baker, 2001), using only isolated molecules (enzymes, transport proteins) rather than the functionality of living cells and the complex feedback mechanisms that are inherent to in vivo assays in addition to the disadvantages such as time demand and costs.

We developed an in vitro bioassay based on thyroid hormone dependent cell proliferation, the so-called T-Screen, which can be used to specifically study interference of xenobiotics with $T_3$-receptor interaction at the cellular level. This T-Screen is based on the thyroid hormone dependent cell growth of a rat pituitary tumour cell line (GH3) in serum-free medium that was initially described as a model to study basic thyroid hormone dependent cell physiology (Hohenwarter et al., 1996). In addition to the protocol development and further optimizing steps for environmental samples, reduction of AlamarBlue™ was incorporated into the assay as a measure of cell growth. In the T-Screen compounds are tested both in the absence and in the presence of thyroid hormone ($EC_{50}$ concentration of $T_3$) to test for agonistic and antagonistic potency.

Some known agonists and antagonists that serve as model compounds, compounds with in vivo effects on the thyroid hormone dependent metamorphosis of amphibians and some sediment extracts were tested for their potency to alter thyroid hormone dependent cell growth (Table 1). Sediment extracts

Table 1
Compounds tested in the T-Screen

| Category | Compound [CAS RN] | Reference |
|---|---|---|
| I | Thyroid hormones or compounds resembling the structure of thyroid hormones | |
| | $T_3$ (3,3′-5-triiodothyronine) [6893-02-3] | |
| | $T_4$ (3,3′,5,5′-tetraiodothyroxine) [51-48-9] | |
| | Triac (3,3′-5-triiodothyroacetic acid) [51-24-1] | |
| | Tetrac (3,3′,5,5′-tetraiodothyroacetic acid) [67-30-1] | |
| II | Compounds that were reported to compete with $T_3$ in binding to isolated nuclear thyroid hormone receptors | |
| | Mefenamic acid [61-68-7] | Topliss et al. (1988) |
| | Furosemide [54-31-9] | Topliss et al. (1988) |
| | Lithium chloride [7447-41-8] | Bolaris et al. (1995) |
| III | Compounds that were reported to interfere with the thyroid hormone dependent process of amphibian metamorphosis | |
| | Atrazine [1912-24-9] | Stoker et al. (2000) |
| | Cypermethrin [52315-07-8] | Maiti and Kar (1997) |
| | 2,4-D [94-75-7] | Rawlings et al. (1998) |
| | *p,p′*-DDE [72-55-9] | O'Connor et al. (1994) |
| | *p,p*-DDT [50-29-3] | Herfenist et al. (1989) |
| | Dieldrin [60-57-1] | Cheek et al. (1999) |
| | Dimethoate [60-51-5] | Herfenist et al. (1989) |
| | Diuron [330-54-1] | Schuytema and Nebeker (1998) |
| | Fenitrothion [122-14-5] | Herfenist et al. (1989) |
| | Fentinchloride [639-58-7] | Fent and Meier (1994) |
| | Hexachlorobenzene [188-74-1] | Raaij et al. (1993) |
| | Malathion [121-75-5] | Fordham et al. (2001) |
| | Pentachlorophenol [87-86-5] | Rawlings et al. (1998) |
| | Permethrin [52645-53-1] | Maiti and Kar (1997) |
| | Triallate [2303-17-5] | Rawlings et al. (1998) |
| IV | Sediment extracts from polluted areas | |
| | Dommel | LOES (Vethaak et al., 2002) |
| | Hoogeveen | Observed malformations in frogs |
| | North Sea Channel | LOES (Vethaak et al., 2002) |
| | Terneuzen | LOES (Vethaak et al., 2002) |

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were incorporated in this study as sediments are the final sink for many persistent chemicals and may thereby form a source of exposure to thyroid hormone disrupting compounds especially for organisms in aquatic food chains.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were of >98% purity. 3,3′-5-triiodothyroacetic acid (Triac) and 3,3′,5,5′-tetraiodothyroacetic acid (Tetrac) were a kind gift of Theo Visser (Erasmus Universiteit, Rotterdam). Cypermethrin, 2,4-D, *p,p*′-DDT, dieldrin, dimethoate, diuron, fenitrothion, fentinchloride, hexachlorobenzene, malathion, pentachlorophenol, permethrin, triallate were a kind gift of M. Gemeiner (University of Veterinary Medicine, Vienna, Austria). $T_3$, $T_4$, 5,5′-diphenylhydantoin (DPH), mefenamic acid, furosemide, ethanolamine, sodium selenite, human apotransferrin and bovine serum albumin were obtained from Sigma Chemical Company (St. Louis, MO, USA). Lithium chloride was obtained from Merck Chemical Company (Darmstadt, Germany). Atrazine and *p,p*′-DDE were obtained from Riedel-de Haen (Seelze, Germany). Dimethylsulfoxide (DMSO, 99.9% pure) was obtained from Janssen Chimica (Geel, Belgium), bovine insulin from GibcoBRL (Paisley, Scotland) and AlamarBlue$^{TM}$ from Biosource (Camarillo, USA). Thyroid hormone stocks were prepared in 0.1 M NaOH and stored at −20 °C prior to use. Compounds and environmental extracts for exposure of cells were dissolved and subsequently diluted in DMSO or ethanol where appropriate.

### 2.2. T-Screen procedure

Rat pituitary tumour cells (GH3) were a kind gift of Othmar Hohenwarter (Institute for Applied Microbiology, University of Agriculture, Vienna, Austria). GH3 cells were cultured at 37 °C and 5% (v/v) $CO_2$ in a humid atmosphere in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with HEPES (GibcoBRL, Paisley, Scotland), supplemented with 10% foetal calf serum (GibcoBRL, Paisley, Scotland). Passaging was carried out in 75 cm$^2$ tissue culture flasks every fourth day by releasing the cells from the substrate using 0.1% (w/v) trypsin. Forty-eight hours prior to plating the cells into 96-well microplates (Costar, Cambridge, MA, USA) for the experiment, standard culture medium was changed to serum-free medium PCM as originally described by Sirbasku et al. (1991). PCM medium is a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with HEPES (GibcoBRL, Paisley, Scotland) supplemented with 10 μg/ml bovine insulin, 10 μM ethanolamine, 10 ng/ml sodium selenite, 10 μg human apotransferrin and 500 μg/ml bovine serum albumin. Neither $T_3$ nor $T_4$ was added to PCM medium. Cells were released using a cellscraper (Greiner, Frickenhausen, Germany) and aspirated several times with a pipette as they detach easily and are sensitive to effects of trypsine. Thereafter, cell density was counted, diluted with PCM and seeded into a 96-well microplate in a density of 2500 cells/well in 100 μl medium. PCM medium was used throughout the exposure experiments. Compounds tested in the T-Screen are given in Table 1. Test compounds were diluted in PCM in a 24-well plate in the double desired exposure concentration and 100 μl were added to the respective well. All compounds were tested in the presence of 0.25 nM $T_3$ and in PCM medium without any addition of $T_3$. DPH, a thyroid hormone antagonist, was used as a control compound (Mann and Surks, 1983). All compounds were tested in triplicate including the vehicle control. Plates were incubated for 96 h. As cells being tested grow, innate metabolic activity was measured using AlamarBlue$^{TM}$ (Nakayama et al., 1997). In the AlamarBlue$^{TM}$ assay resazurin is reduced from an almost non-fluorescent oxidized form into its highly fluorescent reduced form resorufin. Experiments to determine optimal dilutions of AlamarBlue$^{TM}$, incubation time and cell density to obtain linear relationships between fluorescence and cell densities were performed according to the manufacturers leaflet. Four hours after addition of AlamarBlue$^{TM}$ the experiment was terminated and plates were analysed in the manner outlined in the corresponding assay procedure. GH3 cells are not able to divide in PCM without $T_3$ but keep basal activity. Concentrations of test compounds that inhibited basal turnover of AlamarBlue$^{TM}$ of GH3 cells cultured in PCM without $T_3$ were considered to be cytotoxic.

To test whether GH3 cells have biotransformation activity of CYP1A1 or CYP2B and therefore the intrinsic ability to form thyroid hormone active metabolites, cells were incubated with 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) or phenobarbital, and thereafter ethoxyresorufin (EROD) and pentoxyresorufin (PROD) activities were analysed (Sanderson et al., 1996).

### 2.3. Preparation of sediment extracts

Sediment extracts were obtained from four known polluted sites in The Netherlands, namely the North Sea Channel close to IJmuiden, the Westerschelde close to Terneuzen, the Dommel river close to Eindhoven – all three also studied in the Dutch LOES project (Vethaak et al., 2002). In addition sediment was collected from a small channel close to Hoogeveen, where malformed amphibians have been observed. Apolar fractions of 1 g sediment were obtained as previously described (Legler et al., 2002), and cells were exposed to extracts equivalent to 250 mg sediment.

### 2.4. Data analysis

A complete $T_3$ standard curve was included in each assay. The standard curve was fitted using a cumulative fit function using Slide Write 4.0 for windows. Cell proliferation

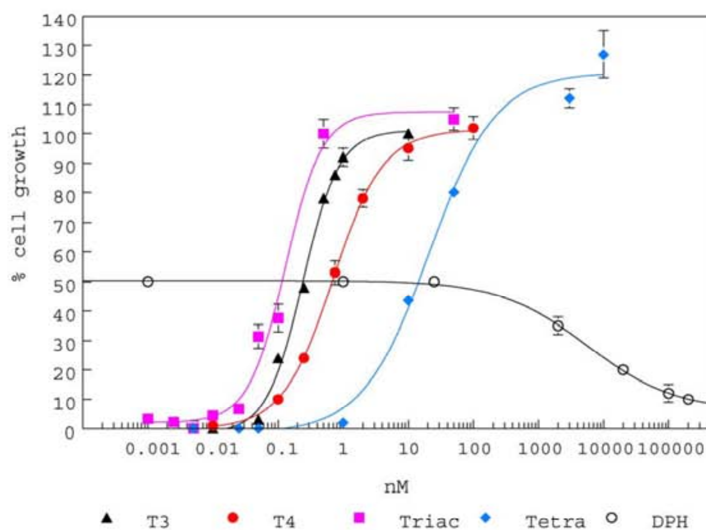


Fig. 1. Dose–response curves for $T_3$, $T_4$, Triac, Tetrac and DPH. Cell growth in the presence of DMSO without $T_3$ was set to 0% and the maximum of cell growth in the presence of 10 nM $T_3$ was set at 100%. DPH was tested in the presence of 0.25 nM $T_3$.

was expressed relative to the maximum response observed at 10 nM $T_3$, which was set at 100% induction. The response for the solvent control was set at 0%. The $EC_{50}$ or $IC_{50}$ for the compounds was calculated by determining the concentration at which 50% of the maximum cell proliferation, respectively, 50% of the maximum decrease of cell growth in the presence of $T_3$ was reached using the cumulative fit equation. The relative potency of compounds was calculated by dividing the $EC_{50}$ of $T_3$ by the $EC_{50}$ of the compound of interest.

Table 2
T-Screen growth effects of GH3 cells resulting from exposure to $T_3$, $T_4$, Triac, Tetrac and DPH

| Compound | *n* | $EC_{50}$ (S.E.M.) | Relative potency [b] |
|---|---|---|---|
| $T_3$ | 17 | 191 pM (23) | 1 |
| $T_4$ | 3 | 654 pM (239) | 0.29 |
| Triac | 3 | 141 pM (12) | 1.35 |
| Tetrac | 3 | 2448 pM (134) | 0.07 |
| DPH | 4 | 46 μM[a] (23) | na |

*n* = Number of independent experiments, na = not applicable.
[a] $IC_{50}$.
[b] Calculated as ratio of $EC_{50}$ ($T_3$)/$EC_{50}$ (compound).

## 3. Results

There was no cell proliferation in PCM medium without $T_3$ and basal metabolic activity as indicated by reduction of AlamarBlue$^{TM}$ remained low (approximately 1000 RFU) unless concentrations of tested compounds caused cytotoxicty and RFUs became as low as 400. Maximum induction after exposure to $T_3$ resulted in an eight-fold increase of RFUs compared to unstimulated cells. Incubation with TCDD or phenobarbital did not result in an induction of EROD or PROD compared to controls (data not given). Dose–response curves of $T_3$, $T_4$, Triac, Tetrac and DPH are given in Fig. 1, and $EC_{50}$ values, induction factors for $T_3$, $T_4$, Triac and Tetrac, respectively, the $IC_{50}$ value for DPH are given in Table 2. Triac was more potent than the active form $T_3$ with a relative potency of 1.35. The potency of $T_4$ was 0.29 and that of Tetrac 0.07 compared with $T_3$. DPH exposure in the presence of 0.25 nM $T_3$ resulted in decreased cell growth that was as low as 40% of controls at 200 μM DPH. No effects on basal metabolic activity of GH3 cells in the absence of $T_3$ were observed at this concentration.

Fentinchloride significantly increased cell growth (one-way ANOVA, $P < 0.01$) both in the presence and absence of $T_3$ at concentrations up to 2 nM, followed by a significant decrease in cell growth (one-way ANOVA, $P < 0.01$) with concentrations higher than 50 nM resulting in cytotoxicity (Fig. 2). All other compounds (Table 1) gave no response in concentrations up to cytotoxic levels that were observed at 10 μM or higher (data not shown).

Apolar sediment extracts from the Dommel (34%) and Terneuzen (14%) decreased cell growth in the presence of 0.25 nM $T_3$, whereas the extract from location Hoogeveen increased cell growth (26%). The sediment extract from the North Sea Channel had no effect (Fig. 3).

## 4. Discussion

The results of this study demonstrate that the thyroid hormone dependent growth of GH3 cells as analysed by the reduction of AlamarBlue$^{TM}$ can be used as a bioassay to detect

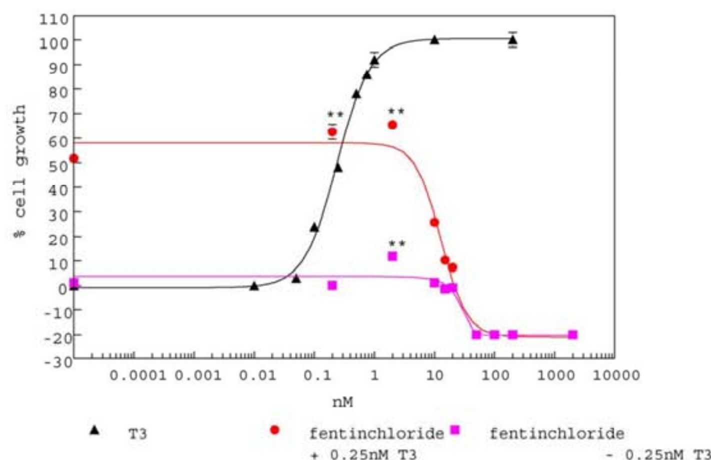


Fig. 2. Dose–response curve for fentinchloride. Statistical evaluation was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Bonferoni test that controlled Type I experimentwise error. The symbol (★★) indicates $P < 0.01$; significance levels are only shown for increase of cell growth compared to the corresponding control.

and quantify interference of compounds with thyroid hormone action at the cellular level. Compounds that resemble the structure of thyroid hormones such as Triac and Tetrac, or that are known to decrease the entry of $T_3$ into cells (DPH) increased respectively decreased the cell growth.

Thyroid hormones induced cell growth, with the rank order of potency Triac > $T_3$ > Tetrac > $T_4$. This order parallels the affinities of these compounds for nuclear thyroid hormone receptors (Samuels et al., 1979; Halpern and Hinkle, 1984).

Exposure to DPH resulted in a dose dependent decrease of cell growth in the presence of $T_3$ (Fig. 1), whereas in the absence of $T_3$, basal metabolic activity in GH3 cells was unaltered at the same concentrations (data not shown). This indicates that inhibition of cell proliferation was not due to cytotoxicity. DPH is known to decrease the entry rate of $T_3$ into cells, and this effect was shown to be fully reversible up to 200 μM DPH (Zemel et al., 1988) and increasing concentrations of $T_3$ can counteract the effects of 200 μM DPH

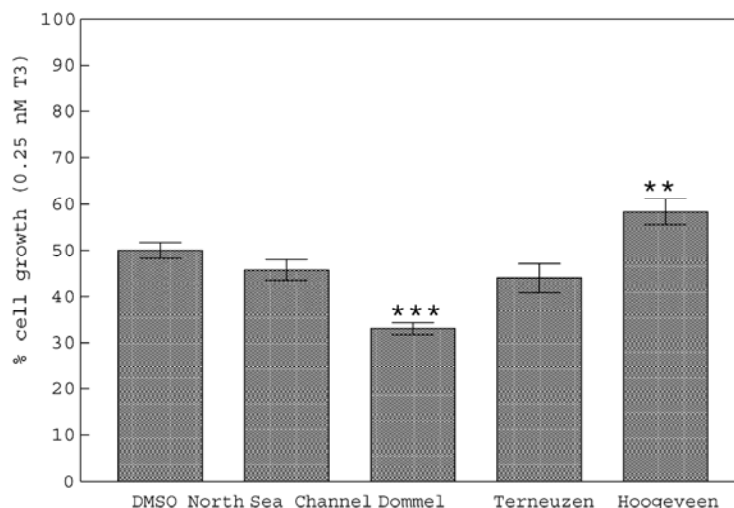


Fig. 3. Effects of apolar sediment extracts on thyroid hormone dependent cell growth of GH3 cells in the presence of 0.25 nM $T_3$ ($EC_{50}$). Cells were exposed to extract of 0.250 g sediment. Cell growth in the presence of DMSO without $T_3$ was set at 0%, and the maximum of cell growth in the presence of 10 nM $T_3$ was set at 100%. Statistical evaluation was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Bonferoni test that controlled Type I experimentwise error. The symbols (★★) and (★★★) indicate $P < 0.01$ and $P < 0.001$, respectively.

and result in normalisation of cell growth (Smith and Surks, 1984; Gingrich et al., 1985).

None of the three tested compounds, chosen because of their ability to inhibit binding of $T_3$ to isolated nuclear receptors, namely mefenamic acid and furosemide (Topliss et al., 1988), and lithium chloride (Bolaris et al., 1995), gave any response in the T-Screen up to concentrations causing cytotoxicity as indicated by the inability of cells to produce fluorescent products. The results for mefenamic acid and furosemide from in vitro binding studies to isolated nuclear receptors may, therefore, not be representative of a functional cellular system where compounds have to be able to enter a cell and activate or inhibit the receptor. Lithium chloride had effects on $T_3$ binding to isolated nuclei from rat cerebral hemispheres after in vivo exposure in rats (Bolaris et al., 1995), a situation that cannot be directly compared with an in vitro cell system based on a different cell type.

A wide variety of compounds has been tested because their ability to interfere with the in part thyroid hormone dependent process of amphibian metamorphosis has been reported in the past from in vivo studies (for compounds and references, see Table 1), but none of them showed any effect on growth of GH3 cells up to concentrations that were cytotoxic. Cellular uptake and binding of thyroid hormone to its receptor is only one step in a cascade that includes synthesis, transport, activation and metabolisation of thyroid hormone needed for amphibian metamorphosis. Also, a range of other factors, such as growth hormone, corticosteroids, temperature or food availability, play a role (Leips and Travis, 1994; Scott, 1994; Hayes, 1995). Also, other compounds that were tested based on their thyroid hormone disrupting potency in vivo in other species induced no response up to cytotoxic concentrations, suggesting their mode of action is disrupting thyroid hormone homeostasis on an extracellular level rather than inducing $T_3$-like effects.

In addition to the compounds interfering with amphibian metamorphosis, fentinchloride was tested as exposure of fish (Fent and Meier, 1994) resulted in effects that were almost identical to what we have observed in tadpoles after exposure to PCB 126, a compound that is known to disrupt thyroid hormone homeostasis (Gutleb et al., 1999). The growth curve of GH3 cells in the presence of fentinchloride at low concentrations followed the classic hormetic curve (Fig. 2), a commonly observed growth-related phenomenon (Calabrese, 2002). In addition, concentrations that result in a decrease of cell growth in the presence of $T_3$ do not cause such a decrease in the absence of $T_3$. To our best knowledge, no reports on the mechanism of the thyroid hormone disrupting ability of fentinchloride have been published so far, but as effects were seen only in the presence of $T_3$, it may be an indirect effect rather than a direct effect on the receptor.

Extracts from Dommel and Terneuzen reduced cell growth in the T-Screen in the presence of 0.25 nM $T_3$ (Fig. 3). Both locations are heavily polluted with wastewater effluent and industrial compounds such as brominated flame-retardants (Vethaak et al., 2002). The sediment from Hoogeveen had high concentrations of PAHs such as fluoranthene and pyrene up to 1800 μg/kg dry weight, respectively, 1100 μg/kg dry weight (Torenbeek, pers. comm.) and increased the cell growth in the T-Screen. Sediments from The Netherlands have recently been shown to contain principles with thyroid-hormone displacing ability in the $T_4$-TTR assay (Houtman et al., 2004). To our best knowledge, this is the first report in which sediment extracts are reported to influence thyroid hormone receptor dependent processes in living cells. Although the thyroid hormone disrupting principles in the polluted sediments are not identified yet, these results are very promising for further research on the presence of thyroid hormone disrupting compounds in the environment. Comparison between induction of cell proliferation in $T_3$-dependent GH3-cells and $T_3$-independent cells such as CHO or HEK cells can indicate whether this induction is thyroid hormone receptor dependent.

In combination with test systems such as the in vitro thyroid peroxidase assay and deiodinase assays (as summarized in DeVito et al., 1999), and in vitro $T_4$-TTR-competition binding studies (Lans et al., 1993) that all are based on isolated target molecules and different mechanisms the T-Screen is rather based on a functional cell system and is able to specifically predict and identify the $T_3$-specific thyroid hormone disturbing potency of single compounds, mixtures thereof or environmental extracts. A pre-selection of compounds based on in-vitro assays can finally also result in reduction and refinement of animal experiments.

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# 7

## Summary and General Discussion

*Rana temporaria*
(photo courtesy of Jane Burton, http://www.warrenphotographic.co.uk)

# SUMMARY AND GENERAL DISCUSSION

## Overview of the experimental approach

The overall objective of this thesis was to detect and study interference of environmentally relevant concentrations of contaminants with thyroid hormone (TH)-dependent amphibian development during sensitive stages. Throughout the thesis life stages of amphibian development were scored according to (Nieuwkoop and Faber, 1975) (NF stages). Amphibian metamorphosis is long known to be TH-dependent and it has been hypothesized that alteration of TH homeostasis (Carey and Bryant, 1995; Kloas, 2002; Freeman et al., 2005) may be in part responsible for worldwide occurring amphibian declines (Wake, 1991; McCoy, 1994; Pechmann and Wilbur, 1994; Sarkar, 1996; Houhlahan et al., 2000; Williams, 2004). A growing number of xenobiotics used as pesticides, herbicides and industrial compounds are suspected to have deleterious effects on development by disrupting hormone sensitive processes including TH mediated processes. The present thesis aims at investigating whether environmentally relevant concentrations of some of these compounds may indeed add to the disruption of TH-dependent amphibian metamorphosis

This overall objective of the thesis was translated into the following three research questions, all focussing on TH dependent amphibian development:

1) What are the most sensitive periods for disruption of amphibian development by toxic compounds?
2) Are effects to be expected at environmentally realistic exposure concentrations?
3) What are the best methods to study and predict possible disruption of thyroid hormone dependent amphibian development?

In order to answer these research questions, *in vivo* studies were performed applying different exposure routes during different life stages of amphibians (see Figure 1.4 on page 26). Based on the findings in this thesis two new *in vivo* bioassays were developed focussing on possible adverse effects of compounds on thyroid hormone dependent development. In one assay (Chapter 2 and 3) exposure occurred via the water phase during very early embryonic developmental stages only. In the other assay (Chapter 4 and 5) the tadpoles were orally exposed starting before the onset of thyroid hormone dependent metamorphosis. These assays were optimized, and validated with PCB-standards and applied for diluted extracts of polluted sediments.

Technical mixtures of PCBs, single PCB congeners or apolar sediment extracts from known polluted sites in the Netherlands were used as test compounds to validate the newly developed assays and to obtain insight in whether these tests could detect adverse effects at environmentally relevant concentrations of TH disrupting compounds (research question 2).

In addition to exposure of the tadpoles themselves the search for the most sensitive period for disruption of amphibian development by toxic compounds (research question 1) also included experiments with exposure of the eggs before fertilization by orally dosing female adult frogs as persistent lipophilic compounds are passed on to the eggs (Chapter 2).

In a search for the most sensitive test protocol (research question 3), previously unexposed embryos were exposed via the water-phase during their 96-hour development into free swimming and feeding larvae. The exposed tadpoles were either sacrificed immediately after this 96-hr exposure period (standard FETAX protocol) or they were followed in their development without further exposure until tadpoles successfully completed metamorphosis (prolonged-FETAX) (Chapter 2 and 3). Optimizing the test protocols it was also discovered that the use of laboratory gloves in aquaria with tadpoles result in drastic confounding effects, which unintentionally biased the results of toxicological research using tadpoles as experimental animals (Chapter Intermezzo).

In addition to i) early exposure via orally dosing female adult frogs, and ii) exposure via the water phase during very early embryonic developmental stages only, a third exposure regimen studied in the present thesis focused on iii) late exposure of tadpoles starting before the onset of thyroid hormone dependent metamorphosis. This was performed with previously unexposed animals, orally dosed via their diet just before the onset of metamorphosis starting at NF stage 51 either for a 10-day period only or continuously until successful metamorphosis (Chapter 4). In addition, in the search for the most sensitive test protocol, aiming at refinement and thereby reduction of animal experiments, a further improvement of the *in vivo* assays was achieved by the synchronization of the animals just before this onset of metamorphosis. This resulted in the newly developed Synchronized Amphibian Metamorphosis Assay (Chapter 5).

Finally, in order to add to all the principles of reduction, refinement and replacement (3 R's) of animal experiments, in our search for optimal testing strategies, an *in vitro* assay based on thyroid hormone dependent cell growth of a rat pituitary cell-line (GH3) was adapted to pre-screen compounds for their functional thyroid hormone mimicking potencies (Chapter 6).

Based on the results of the experiments described in this thesis the answers to the 3 research questions are discussed below.

***1) What are the most sensitive periods for disruption of amphibian development by toxic compounds?***

Developing organisms including humans and rodents, are particularly sensitive for exposure to EDCs during critical windows (Selevan et al., 2000; Slotkin et al., 2005a,b), in which exposure induces long-term changes in reproductive as well as non-reproductive organs, including persistent molecular alterations (Carruthers and Foster, 2005). Contrary to mammals nothing has been specifically published on critical windows of amphibian development.

In this thesis the search for the most sensitive period for disruption of amphibian development by toxic compounds focuses on three exposure regimens. These included:

i. exposure of the eggs before fertilization by orally dosing female adult frogs (Chapter 2),
ii. exposure of the very early embryonic developmental stages via the water phase (chapter 2 and 3),
iii. exposure of previously unexposed tadpoles via their diet starting just before the onset of metamorphosis, either for a 10-day period or continuously until successful metamorphosis (chapter 4 and 5).

In the prolonged-FETAX adverse effects were detected at exposure concentrations of 300 nM PCB 77 during 4 days (Chapter 3) In the Synchronized Amphibian Metamorphosis Assay exposure to a concentration of 16 nM Clophen A50 (dose via the water calculated per l of water) during 60-days significantly delayed duration until successful metamorphosis (Chapter 5). Also the very early exposure via the mother animal with the low internal dose of 800 ng/g lipid of the PCB mixture Clophen A50 resulted in significant effects in the following early embryonic stages (mortality, rate of malformations) but also resulted in long-term effects such as reduction of froglets body weight (Chapter 2). Up to two thirds of the total body burden of PCBs in adult females is transported into the egg resulting in significant exposure of the embryos (Kadokami et al., 2004) showing the relevance of this exposure route. Furthermore alterations of female physiology such as PCB-induced lowered retinoid and thyroid hormone plasma levels will consecutively also influence egg composition (Fernie et al., 2000; Boily et al., 2003) and thereby embryonic developmental processes, as early embryonic stages are dependent on hormones and vitamins present in the egg. By exposing females also pre-fertilization events are included in the experimental setting, adding an element that is not often covered in reproductive toxicology.

Embryonic stages are especially vulnerable to hormonal imprinting. Hormonal imprinting develops at the first encounter between the

target hormone and the receptor (Karabélyos and Csaba, 1998). Excess of hormones or exogenous compounds that are able to bind to the maturing receptor lead to faulty imprinting that results in disturbance of normal receptor function, such as receptor densities, abnormal cellular responses and in deviation of physiological, morphological and even behavioural parameters later in life (Csaba and Inczefi-Gonda, 1999; Cupp et al., 2003; Uzumcu et al., 2004). Other factors that influence stage specific sensitivity to toxic compounds are related to the changing physiology of different life stages such as extent of organogenesis, cellular differentiation, and enzyme activity and are dependent on the stage of development as shown for different compounds and amphibian species (Cardellini and Ometto, 2001; Harris et al., 2000; Schuytema and Nebeker, 1998; Hatch and Burton, 1998; Richards and Kendall, 2002).

Oral exposure starting at NF stage 51 (in the not-synchronised tadpoles) significantly increased mortality (Chapter 4) whereas no such mortality was observed when the experiment was started with synchronised tadpoles of the later NF stage 54 (Chapter 5). Apparently in the NF stages 51-53 tadpoles are relatively sensitive for general toxic effects that lead to mortality. The synchronised tadpoles were halted in NF stage 54 for already some time and exposure started already before the animals were allowed to continue their development. They only experienced more specific effects on the process of metamorphosis.

Results of the present thesis indicated that both embryonic life-stages as well as later developmental stages just before the onset of metamorphosis are very susceptible to the TH disrupting compounds at the moment of tissue formation and tissue remodeling respectively.

### *2) Effects to be expected at realistic concentrations?*

In exposed adult female *Xenopus laevis* a liver concentration for the sum of seven PCB congeners (IUPAC nr. 28, 52, 101, 118, 138, 153, 180) of 800 ng/g lipid was found in the lowest dose group which resulted in significant lowered body weight of metamorphosed offspring (Chapter 2). This concentration in the adult experimental animals is 2 two 5 times lower than what is reported for frogs from the wild (Angermann et al., 2002; Gilliland et al., 2001; Fontenot et al., 2000; de Solla et al., 2002).

Concentrations of PCB 126 inducing significant effects in the prolonged-FETAX were derived on the basis of dioxin-like toxic equivalency concentrations known to be present in pore-water (Murk et al., 1996). This reflects the environment in which an amphibian embryo is exposed in close contact with sediment (Karasov et al., 2005). Exposing the embryos to the apolar extracts of only 250 mg sediment from known polluted sites in the Netherlands dissolved in 10 ml water resulted in significant effects

on body weight, larval period, percentage of metamorphosed froglets, stage distribution of not metamorphosed tadpoles, and accredited penalty points) (chapter 3).

The lowest concentration of the technical PCB mixture Clophen A50 (0.2 mg/kg feed) inducing significant effects after the 60 days exposure period (chapter 5) is approximately 30% lower than the concentration in phytoplankton, the natural food for tadpoles from many amphibian species, from several water bodies in Europe (van Hattum et al., 1992; Leonards et al., 1997; Berglund et al., 2001). Finally, the extract of only 1 g of contaminated sediment mixed with 1 kg of food was able to induce a significant delay in development that is reflected in dose-related increase in cumulative penalty points in the Synchronized Amphibian Metamorphosis Assay (Chapter 5). Calculated into water concentration this was equivalent to only 0.0025 g of sediment/l of water.

Therefore the results of the present thesis clearly demonstrate that both environmentally relevant PCB concentrations found at polluted sites throughout Europe, as well as environmentally relevant concentrations of mixtures of the wide range of apolar compounds present in polluted sediments are able to significantly alter amphibian embryo and tadpole development.

### *3) The best methods to study and predict possible disruption of thyroid hormone dependent amphibian development*

Possible disruption of thyroid hormone-dependent amphibian development can be studied directly with *in vivo* experiments and, after sufficient validation, probably also be predicted based on *in vitro* experiments. Principally there are three ways to study effects of early life-stage exposure of embryos and tadpoles of amphibians namely either a) by dosing embryos indirectly via dosing the females prior to egg-laying (Chapter 2), b) by exposing developing embryos via the water (FETAX; prolonged-FETAX) (Chapter 2 and 3) or c) by exposing tadpoles via the food (standard or Synchronized Amphibian Metamorphosis Assay) (Chapter 4 and 5).

Exposing embryos via adult females that were dosed with environmental relevant concentrations of Clophen A50 resulted in significant effects (Chapter 2). Although this approach has the potential to detect teratogenic effects of compounds and indirect effects via the mother animal, the number of experiments applying exposure via adult frogs is very limited (Pickford and Morris, 2003; Kadokami et al., 2004) compared with studies directly exposing embryos and tadpoles (Hatch and Burton, 1998; Harris et al., 2000; Jelaso et al., 2003, 2005; Relyea, 2005; Relyea and Mills, 2001; Richards and Kendall, 2002; Savage et al., 2002; Zhou et al., 2004). Of course performance of such an indirect exposure

experiment is more complicated and elaborates the number of experimental animals used because it includes exposure of adult frogs as well.

The second option is exposing the embryos during early life-stages starting from NF stage 13 until NF stage 46 (FETAX and prolonged-FETAX) (Chapter 2 and 3). In that case experiments start with healthy eggs of which the condition is not impaired via possible indirect effects (e.g. reduced vitamin A and thyroid hormone levels) in the female. The research as presented in this thesis reveals that very early exposure of *Xenopus laevis* embryos up to NF stage 46 for 96 hours with any of the tested compounds according to the generally applied FETAX does not induce any acute toxic effects (Chapter 2 and 3). This is in accordance with the results of Zhou and colleagues (2004) showing that Aroclor 1254 had no effect in the FETAX and the lower chlorinated mixture Aroclor 1221 significantly increased mortality only at 10 mg/L, a concentration that surpasses maximum solubility. The fact that delayed significant effects on survival, condition and the process of metamorphosis were found in the prolonged-FETAX (Chapter 2 and 3), reveals that current test schemes using short-term early life stage tests such as the FETAX may underestimate the risk of compounds with low acute toxicity. Thyroid hormone receptors mediate both early and late developmental programs of metamorphosis (Schreiber et al., 2001) and hormonal imprinting as explained for research question 2, may

therefore play a role for the observed effects. As the prolonged-FETAX includes observation of the FETAX-effects as well, it makes much more efficient use of the exposed experimental animals.

The third possibility is to expose tadpoles via the diet, the most important route for lipophilic compounds under natural conditions. In this thesis the Synchronized Amphibian Metamorphosis Assay is proposed as an improvement of the standard metamorphosis assay. All animals can be kept available as a homogenous group in NF stage 54 until used. This is in contrast to the classical metamorphosis assay where the fastest and slowest developing animals cannot be used because otherwise the group of animals would be too variable reducing the statistical power of the experiment. This approach allows easy 'loading' the animals with the compounds to be studied in the period before the thyroid hormone surge in a developmental stage (NF stage 54) during which they are not so sensitive for direct toxicity (Chapter 5). An additional advantage is that Synchronized Amphibian Metamorphosis Assay can be performed in 60 days instead of 76 days for the standard metamorphosis assay starting at NF stage 51 (Chapter 4 and 5).

Altogether the results of the present thesis illustrate that the two newly developed assays, namely the prolonged-FETAX and the Synchronised Amphibian Metamorphosis Assay are the most

sensitive and relevant in vivo test protocols for detecting TH disrupting effects on amphibian development and metamorphosis.

Finally, in order to add to the principles of reduction, refinement and replacement (3 R's) of animal experiments, an *in vitro* assay based on thyroid hormone dependent cell growth of a rat pituitary cell-line (GH3) was adapted in our search for optimal testing strategies to pre-screen compounds for their functional thyroid hormone mimicking potencies (Chapter 6).

The responses in the *in vitro* T-screen used in this thesis (Chapter 6) were comparable to the potency of these extracts to alter tadpole metamorphosis in the Synchronized Amphibian Metamorphosis Assay (chapter 5 and 6). This shows the potential of the T-Screen as a screening tool for thyroid hormone related effects. This assay and TRα en TRβ -specific reporter gene assays presently under development (Schriks et al., subm.) will be invaluable tools to identify compounds or environmental extracts with TH disrupting potency. This will help minimizing the need for animal systems for this purpose.

Overall the results described in this thesis indicate that a combination of the *in vitro* T-screen with the *in vivo* prolonged-FETAX and the Synchronized Amphibian Metamorphosis Assay can form a valuable contribution to the requested test battery (EDSTAC,

1998; OECD, 2005), which can identify compounds with TH-disrupting potency.

***Possible future developments***

Principally endocrine systems can be affected in various ways including effects on the processes of synthesis and release, transport to the tissue of action, uptake into the cell, receptor-binding, metabolic steps and feedback mechanisms. To unravel mechanisms of endocrine disruption is especially complex when the physiological function under study is supported by multiple endocrine systems that exhibit receptor crosstalk, variation of gene expression relevant for hormone action over time and in different tissue types. In future *in vivo* experiments, compounds interfering with additional hormone systems (i.e. sex steroid, retinoid) should be evaluated alone and in combinations with TH and TH-disrupting compounds. This can reveal further important functions of TH, e.g. indirect effects on the additional hormone systems. In addition this approach may help to fully characterize the mechanisms involved in the disturbance of amphibian development and metamorphosis and to further develop and validate proposed assay schemes.

In the future, additional endpoints using modern molecular biological techniques should be incorporated into current test protocols. Expression of TH receptor mRNA has recently been

suggested as such a sensitive molecular marker for agonism and antagonism of thyroid hormone action (Opitz et al., in press). The fact that animal groups exposed in the Synchronised Amphibian Metamorphosis Assay are more homogenous than in the standard Metamorphosis Assay will assist in the need to reduce variation in the outcome of micro-array experiments thereby reducing the costs by reducing the number of replicates required (Dowling et al., 1998; Degenkolbe et al., 2005).

Genome information for *Xenopus laevis* is rapidly increasing (http://www.ncbi.nlm.nih.gov/genome/guide/frog; www.xenbase.org) and the commercial genome arrays that are now available offer new possibilities for studying the interaction of EDCs with gene expression (Affymetrix, Santa Clara, USA).

Finally effects on the thyroid hormone system on amphibian tadpole development should be studied in the context of the dynamic natural environment by adding *in situ* approaches to proposed assay protocols.

**Main conclusions**

Exposing amphibian embryos and tadpoles according to both the prolonged-FETAX and Synchronized Amphibian Metamorphosis Assay protocols is a suitable approach to reveal endocrine disrupting

effects of exposure to low doses of lipophilic compounds on the whole development of amphibians from early embryo to froglet.

The standard FETAX-assay does not detect long term effects of early exposure to endocrine disrupting compounds, possibly resulting in a serious underestimation of the risk of PCBs and other apolar environmental extracts.

The *in vitro* T-screen is especially suitable to study the thyroid hormone receptor-mediated aspects of endocrine disruption. This *in vitro* model is a valuable tool for (pre-)screening of compounds and environmental extracts and selecting putative EDCs to be further tested in *in vivo* systems.

Exposure of amphibian larvae to environmentally relevant concentrations of PCBs and apolar sediment extracts via relevant exposure routes resulted in significant adverse developmental effects. This strongly suggests that at polluted locations in the Netherlands such as Dommel or Terneuzen, amphibian embryos and tadpoles are exposed to concentrations of xenobiotics that are able to interfere with metamorphosis. Therefore it cannot be excluded that this currently influences local amphibian populations.

Altogether the results of the present thesis demonstrate that, using the newly optimized *in vivo* but also *in vitro* assays, the effects of environmentally relevant concentrations of thyroid hormone disrupting compounds on amphibian development can be easily detected. This corroborates concerns over the role of these environmental pollutants in the presently observed worldwide decline of amphibian populations.

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## Samenvatting

### Opsporen van de effecten van milieurelevante concentraties van schildklierhormoonverstorende stoffen op de ontwikkeling van amfibieën

Persistente organische verontreinigingen zoals PCBs worden ervan verdacht een rol te spelen in de wereldwijde afname van populaties van amfibieën. De schildklierhormoon(SH)huishouding is één van de mogelijke aangrijpingspunten voor milieuvreemde stoffen met een effect op de ontwikkeling van amfibieën, omdat SH bij hen een belangrijke extra rol speelt bovenop de toch al belangrijke rol in de ontwikkeling van alle vertebraten. De metamorfose van kikkervisje tot kikkertje is namelijk afhankelijk van een SH-piek in het bloed net voor de start. Om de mogelijke verstoring van de ontwikkeling van amfibieën te onderzoeken zijn twee bioassays ontwikkeld: één met blootstelling van zeer jonge ontwikelingsstadia en één met blootstelling net voor de SH-piek. De testen werden geoptimaliseerd, gekarakteriseerd met PCBs als standaarden, en toegepast voor het testen van verdunde extracten van verontreinigd sediment. Een *in vitro* assay werd opgezet als screenings test voor SH-verstorende stoffen door gebruik te maken van cellen die voor hun groei SH nodig hebben in hun medium.
Het onderzoek dat gepresenteerd wordt in dit proefschrift laat zien dat de momenteel veel gebruikte test voor vroege ontwikkelingsverstoring door stoffen, de zogenoemde FETAX-test, geen effecten kan aantonen van de geteste PCBs en de apolaire sediment extracten tijdens de 96-uur durende test periode. De in dit promotieonderzoek nieuw ontwikkelde verlengde-FETAX toont aan dat er wel degelijk verstoringen worden veroorzaakt door zelfs veel kleinere hoeveelheden van dergelijke stoffen. Effecten zijn gevonden op totaal lichaamsgewicht, tijdsduur van de metamorfose en het percentage van de dieren dat succesvol de metamorfose doorloopt. In de zogenoemde gesynchroniseerde metamorfose test met amfibieën worden alle dieren in hun ontwikkeling gestopt net voor de SH-piek, in stadium NF stage 54. Het voordeel is dat de experimentele groep altijd beschikbaar is en relatief homogeen. Zo kunnen significante ontwikkelingseffecten van aan het voer toegevoegde PCBs en sediment extracten al worden aangetoond met relatief weinig dieren.
De aanpak waarbij zowel effecten op zeer jonge embryos (verlengde-FETAX) als kikkervisjes (gesynchroniseerde metamofose test) worden getest zal uiteindelijk beter het risico van milieucontaminanten voor zich ontwikkelende organismen kunnen weergeven dan de huidige korte termijn test met hoge blootstelling via alleen het water.
De negatieve effecten van de sterk verdunde apolaire sediment extracten suggereren dat SH-verstorende stoffen in het aquatische milieu aanwezig zijn die inderdaad een risico vormen voor de ontwikkeling van amfibieën en mogelijk andere gewervelde dieren in het wild. De *in vitro* test blijkt bovendien een geschikt hulpmiddel om specifieke vormen van SH-verstoring door toxische stoffen op te sporen.

# CURRICULUM VITAE

Arno Gutleb was born in Klagenfurt, Austria, on May 3, 1962. In 1980 he graduated at the Bundesgymnasium 1 in Klagenfurt. In 1981 he started his study of Veterinary Medicine at the University of Veterinary Medicine (VUW) in Vienna, Austria. During his study he worked for several months at the Landesanstalt für veterinärmedizinische Untersuchungen Ehrental in Klagenfurt and at Biochemie Kundl in Vienna in the registration department. From September 1989 he was employed as a student assistant at the Institute for Medical Chemistry where he started to work as a research assistant after his graduation in July 1990. He was responsible for a project on environmental contaminants in the otter (*Lutra lutra*) that was in part financed by WWF Austria. From 1994 to 1995 he spent 12 months on a shared scholarship of WU and VUW at the Section Toxicology at Wageningen University (WU-Tox) and started with a study on effects of PCBs on amphibians. In 1995 his medical veterinary-thesis entitled "Umweltkontaminanten und Fischotter in Österreich - Eine Risiko-abschätzung für *Lutra lutra*" was approved in Vienna (supervised by Prof. Wolf-Dieter Rausch and Prof. Erich Kutzer) and the price for the best thesis at VUW in 1995 was awarded. In 1997 he received a grant from the Austrian Science Fund (FWF) and started again to work with amphibians at WU-Tox under supervision of Dr. Tinka Murk and Prof. Jan Koeman. Later in 1998 he worked at WU-Tox on an industry-financed position (cell culture), followed by a second grant from FWF before he entered a CEFIC-financed research position at WU-Tox. From 2001 to 2002 he was a senior scientist at the Norwegian Veterinary Institute in Oslo, where he established cell culture techniques to study mycotoxins, followed by a 30 months position as project leader at the Institute for Environmental Studies (IVM) at the Vrije Universiteit Amsterdam. As part of this research project long-term effects of intra-uterine low dose exposure on a variety of endpoints were studied within a EU funded project (COMPARE). Since 2005 he holds a research position at the Department for Production Animal Clinical Science at the Norwegian School of Veterinary Science in Oslo where he is the responsible scientist for a project studying effects of intra-uterine environmentally relevant exposure to EDCs on reproductive and behavioural endpoints (ED-FLAME). In 2005 he attended the Gordon Research Conference on Hormone Action in Development and Cancer, Mount Holyoke College, South Hadley, USA where a Graduate Student-Postdoctoral Fellow Conference Award was awarded to him.
He followed the postdoctoral education in Toxicology in Austria and the Netherlands and is since 2002 a NVT and EUROTOX registered toxicologist.

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## TRAINING AND SUPERVISION PLAN

### *COURSES*

Postdoctoral Course Toxicology, University Vienna, A (1997-2000)
Genetic Toxicology, University of Leiden, NL
Article 9 authorization “Wet op de dierproeven”, The Netherlands

This postdoctoral education lead to registration as fully licenced toxicologist at the Nederlandse Vereniging voor Toxicologie (NVT) and Eurotox.

Stralingsdeskundige 5B, Hogeschool Larenstein, NL
Statistics (SPSS), 1997-1999, Wageningen University, NL
5th Advanced Course on Ecotoxicology, EERO, Texel, NL
European Environment Policy, EERO, Genval, B
GC-Chemstation User Training, Hewlett-Packard, Vienna, A
Successful Presentation, VUW, Vienna, A
Acquisition Training, Boertien and Partners, Amsterdam, NL
How to Negotiate, Manage and Administer Framework 6 Contracts, Hyperion, Ås, N

### *Symposia and Conferences* *(of relevance for this thesis)*

2nd European Conference on Ecotoxicology Amsterdam, NL 1992
Dioxin ´93 Wien, A 1993
Dioxin ´96: Amsterdam, NL 1996
Dioxin ‘98: Stockholm, S 1998
M&T Progress Report Meeting, Ede, NL 1999
11th Annual Meeting SETAC Europe, Madrid, E 2001
Monitoring van stoffen in het aquatische milieu, RIKZ, Den Haag, NL 2002
Keywords in the socio-economic and natural sciences of the environment. Sense Summer Symposium, Leiden, NL 2003
Eurotox 2003, Florence, I 2003
Dioxin 2004, Berlin, D 2004
Hormone Action in Development and Cancer. Gordon Research Conference, Mount Holyoke College, USA 2005
Endocrine disruptors – Occurrence in the bioenvironment and possible effects in animals and humans. Norwegian Academy of Science and Letters, Oslo, N 2005

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