THYROID-DISRUPTING ACTIVITY IN THE SOUTH AFRICAN AQUATIC ENVIRONMENT

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by

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EXECUTIVE SUMMARY

INTRODUCTION

Global concerns have been raised about the possibility that environmental chemicals (mostly man-made) may interfere with the endocrine systems of wildlife and humans (Damstra *et al.,* 2002; OECD, 2006; Zoeller *et al.,* 2012; Bergman *et al.,* 2013; Heindel *et al.,* 2013). These concerns primarily stem from an extensive knowledge base reporting evidence that wildlife, including invertebrates, examples from all vertebrates groups, fish, amphibians, birds and mammals, as well as ecosystem-health in general, may be compromised by endocrine disruptive activities of water-borne man-made chemicals. In addition, increased endocrine related diseases in humans further contribute to this concern (Colborn, 2004; Brenner and Galvez, 2007; Filby *et al.,* 2007; Trudeau and Tyler, 2007; Woodruff, 2007; Heindel *et al.,* 2013). Laboratory experimentation and field studies related reproductive health of wildlife and humans contributed to the formulation of the so-called endocrine disruption hypothesis (Krimsky, 2000; Damstra *et al.,* 2002; Gore, 2007; Diamanti-Kandarakis *et al.,* 2009; Heindel *et al.,* 2013). Concerns mostly focus on the possible adverse effects on the developmental and reproductive biology of organisms inhabiting the aquatic environment, as well as humans as major end-users of water resources. Endocrine disruption of the control and functioning of the reproductive system by far attracted the most attention (Clark *et al.,* 1998; Bogi *et al.,* 2003; Bergman *et al.,* 2013). However, several lines of evidence suggest that environmental chemicals may also interfere/modulate with the normal functioning of the thyroid endocrine system (Brucker-Davis, 1998; DeVito *et al.,* 1999; Leatherland, 1999; Jahnke *et al.,* 2004; Brent *et al.,* 2007; Tan and Zoeller, 2007; Kloas *et al.,* 2009; Jobling *et al.,* 2013).

The thyroid hormones are important for normal growth and development in all vertebrates (fish, amphibians, birds and mammals) (Zoeller and Rovet, 2004; Norris and Carr, 2006; OECD, 2006; Jobling *et al.,* 2013). In addition, thyroid hormones may influence the activity of a wider variety of tissues and biological functions than do any other hormone, for example osmoregulation, metabolism, and post-hatch metamorphosis in fish and amphibians (Norris and Carr, 2006; Blanton and Specker, 2007; Jobling *et al.,* 2013). These iodine-containing hormones are derived from the amino-acid, tyrosine (Norris and Carr, 2006). Tetraiodothyronine (T4, thyroxine) has iodine molecules attached to four carbons while triiodothyronine (T3) lacks an iodine. The regulation of thyroid hormone release and delivery to tissues and cells during development and in the adult represents a complex system, including an extended network of feedback systems (Norris and Carr, 2006; OECD, 2006; Fort *et al.,* 2007; Zoeller *et al.,* 2007; Jobling *et al.,* 2013). Thyroid hormone (TH)

synthesis is controlled by thyrothropin (TSH) released from the pituitary gland as part of the Hypothalamus-Pituitary-Thyroid axis (HPT). The knowledge base on environmental contaminants acting/interfering at several sites within the HPT, including the effects of environmental iodide deficiencies or interference with the uptake of iodide recently expanded (Brucker-Davis, 1998; OECD, 2006; Zoeller *et al.,* 2007a; Kidd *et al.,* 2013).

Given the importance of thyroid hormone for growth and development and several physiological functions in vertebrates, including humans, it seems reasonable to be concerned that environmental contaminants could influence thyroid functioning and signalling, to the extent that populations would be adversely affected (Brucker-Davis, 1998; Hutchinson *et al.,* 2006; Miller *et al.,* 2009). Because of the highly conserved nature of TH life history (synthesis, transport, regulation, and metabolism), environmental contaminants that affect thyroid function in one vertebrate species may well affect the TH functioning in another, including humans (Cheek *et al.,* 1999; Norris and Carr, 2006). Zoeller and Tan (2007) recognized that while the general functionality of the thyroid system may be the same among vertebrates, there are species-specific differences to be considered; for example, the role of thyroid hormones in controlling amphibian metamorphosis is likely to be different in human development. However, applying vertebrate models to answer the question of whether, or how a contaminant interferes with, or modulates thyroid hormone action or any other aspect of the life history of these hormones, does contribute in initial screening programmes.

Considering the important and wide-spread role of thyroid hormones, and the concern that thyroid disruption may be more than a hypothesis, the development and validation of bioassays to identify potential thyroid disrupting contaminants (TDCs) has received much attention recently (DeVito *et al.,* 1999; Degitz *et al.,* 2005; Opitz *et al.,* 2005; OECD, 2006; Fort *et al.,* 2007; Zoeller and Tan, 2007). The aim of the bioassays is to capture the variety of points of modulation within the thyroid endocrine system that may be disrupted by exposure to thyroid disrupting contaminants (TDCs). The one outstanding feature of the thyroid system is the relative large number of regulatory points, which makes it difficult to put together a battery of *in vitro* screens. It is therefore not surprising that the initial focus in screening for general thyroidal functional effects was on whole animal (*in vivo*) systems, for example the *Xenopus* metamorphosis assay (XEMA) (Fort *et al.,* 1999b; Norris and Carr, 2006; OECD, 2006; Kloas *et al.,* 2009), rather than single cell, *in vitro* bioassays. Assays like XEMA however are labour intensive and may include relatively long exposure periods (Degitz *et al.,* 2005; Opitz *et al.,* 2005; Miyata and Ose, 2012). Molecular endpoints (Th dependent gene expression), for example thyroid hormone receptor (TR) mRNA expression (TRα and TRβ) have been quantified following short-term exposure (24 to 48 hours) (Crump

et al., 2002; Opitz *et al.,* 2006a; Zhang *et al.,* 2006; Helbing *et al.,* 2007b). Recently, specific *in vitro* assays were incorporated in testing programmes alongside *in vivo* exposures to address hypotheses regarding specific modes-of-action (Gutleb *et al.,* 2005; Schriks *et al.,* 2006).

Both the Organization for Economic Cooperation and Development (OECD) and the USA-EPA established endocrine disruptor testing and assessment working groups with the mandate to produce detailed review papers (DRPs) and subsequently develop and validate batteries of bioassays for EDC testing (Ankley *et al.,* 1998; EDSTAC, 1998; Fenner-Crisp *et al.,* 2000; Kanno *et al.,* 2003; OECD, 2006; Tan *et al.,* 2007; Tan and Zoeller, 2007). Both organizations published extensive DRPs and the USA-EPA's EDSP (Endocrine Disruptor Screening Programme) is currently validating assays and have recently submitted several first tier assays for external peer review (OECD, 2008b; 2009).

Because of the pivotal regulatory role of thyroid hormone (TH) during amphibian metamorphosis and because of extensive research done at several levels of the biological organization (Shi, 1999; Kloas and Lutz, 2006; Kloas *et al.,* 2009), amphibian metamorphosis has been targeted as an *in vivo* model system to screen for thyroid disrupting activity (Touart, 2002; OECD, 2006; Fort *et al.,* 2007). The USA-EPA's DRP reviewed candidate anuran species and recommended that the African Clawed Frog, *Xenopus laevis* be used in a metamorphosis assay (*Xenopus* metamorphosis assay, XEMA) (Touart, 2002; Degitz *et al.,* 2005; Opitz *et al.,* 2005; Tietge *et al.,* 2005; Fort *et al.,* 2007). The XEMA is basically a morphological assay designed to detect effects of contaminants on metamorphic development, the rationale being that morphological alterations during development reflect effects on TH function. Although the conceptual framework for the XEMA has been developed and the assay recently validated in the USA, Germany, UK and Japan, the need for validation here locally exist, since scientists from South Africa, as a non-OECD member, did not have the means or opportunity to participate in the ring testing programme coordinated by OECD and USA-EPA to validate XEMA. Moreover, it has been recognized that there is a need to investigate additional endpoints as biomarkers for thyroid disruption to be implemented along-side XEMA or as short-term preliminary screening tools. For example, additional biochemical markers for specific pathways as well as molecular tools (differential gene expression of TH receptors) may be used as early warning sign-posts for potential thyroid disruption. There is also a need to study histological (pathological) endpoints in more detail. Although the original XEMA was set-up as a semi-static renewal exposure system, the recommended protocol for XEMA includes a sophisticated flow-through system (Degitz *et al.,* 2005; OECD, 2009). The question is, is the semi-static renewal system (Kloas *et al.,* 2003) acceptable for use in

developing countries and is there simple flow-through systems that could work for screening environmental water?

Apart from using amphibians as models for thyroid disruption screening, the use of developing fish has also been recognized (Crane *et al.,* 2004; 2005; 2006; Blanton and Specker, 2007). Apart from *Xenopus laevis*, several local fish species have the potential to be employed as bioindicator models in EDC studies, such as, *Oreochromis mossambicus*, a species extensively used in the aquaculture industry (Esterhuyse *et al.,* 2008; Esterhuyse *et al.,* 2009; Mlambo *et al.,* 2009; Barnhoorn *et al.,* 2010; Esterhuyse *et al.,* 2010; Swart *et al.,* 2010) and the sharp tooth catfish, *Clarias gariepinus* (Barnhoorn *et al.,* 2004; Marchand *et al.,* 2008; McClusky *et al.,* 2008).

Although South Africa is part of the developing world, it has all the characteristics and human activities associated with a modern developed country. The potential for endocrine disruption is similar to countries in Europe and the Americas. However endocrine disruptor research activity remains limited with a low priority status, compared to developed countries. Initially, the focus was on studying the endocrine disrupting activity (mostly estrogenic activity, feminization) of contaminants like nonylphenol (De Jager *et al.,* 1999) and DDT (Dalvie *et al.,* 2004; Bornman *et al.,* 2007a; de Jager *et al.,* 2009; Van Dyk *et al.,* 2010; Bouwman *et al.,* 2011; Bouwman *et al.,* 2012; Gyalpo *et al.,* 2012). Since the realization that EDCs in South Africa may be a real environmental and human health concern, focus shifted to bioassay development and validation, which culminated in the establishment of a toolbox programme (De Jager, 2010). Initially, *in vivo* models were used to assess estrogenicity (Hurter, 2003; van Wyk *et al.,* 2005; Esterhuyse, 2008; Swart, 2008) and anti-androgenicity (van Wyk *et al.,* 2003), the shift to the *in vitro* bioassays followed (Swart and Pool, 2009a,b; De Jager, 2010; Swart *et al.,* 2010). Although in recent years few studies directly investigated EDC activity in aquatic systems, most of these were focused on reproductive disruption (van Wyk *et al.,* 2005; Bornman *et al.,* 2007b; Barnhoorn *et al.,* 2009). The thyroid disruption programme, developed from the initial mandate to investigate the endocrine disruptor potential of all man-made chemicals, was initiated the USA-EPA and OECD, with the initial focus on the thyroid controlled metamorphosis phenomenon using a South African aquatic frog species, *Xenopus laevis* as model organism (OECD, 2009). Despite the South African origin of *Xenopus laevis*, South African scientists did not have the opportunity to participate in the development and subsequent ring testing of the "*Xenopus* metamorphosis assay, XEMA" to screen for thyroid disrupting contaminants (TDCs) or activity in environmental waters. Through the subsequent explosion of research around the world concerning potential thyroid disruption and the development and validation of the XEMA protocol the gap and need in the local EDC testing capacity is recognized. Apart from

laboratory testing for TDC activity of selected chemicals, the use of XEMA or related short-term molecular assays testing environmental waters is made possible locally since *Xenopus laevis* occur naturally in our aquatic environments. With the knowledge base regarding EDC effects of an aquatic amphibian species like *Xenopus laevis* as well as local freshwater fish models like *Oreochromis mossambicus* growing, the potential to eventually understanding the health and ecological risk (including wildlife and humans) of EDCs in South Africa becomes a real possibility. The overall objective of this project was therefore to set up and evaluate the XEMA approach (OECD, 2009) to identify thyroid disruption activity, evaluate the potential to actually screen environmental water using *Xenopus laevis* tadpoles. At the same time the potential to include freshwater fish species as models (at least in short-term molecular studies) was also investigated.

OBJECTIVES AND AIMS

In light of the need establish an EDC screening and testing programme in South Africa to assess and manage EDCs in our water resources, the following aims, specifically addressing the thyroid disruption issue, were formulated for this study:

AIM 1

To introduce the thyroid endocrine system, concepts of thyroid disruption, summarize and convey the current knowledge regarding and the potential disruption of this system.

AIM 2

To review, set-up and validate the *Xenopus* Metamorphosis Assay (XEMA) (OECD/USA-EPA protocol) for screening and testing water-borne contaminants for thyroid disrupting activity.

AIM 3

To investigate the different aquatic exposure systems: semi-static exposure vs. flow-through exposure.

AIM 4

To describe potential endpoints for thyroid disruption, including morphological, histological, molecular (gene) and other biochemical endpoints.

AIM 5

To report on the available short-term *in vitro* bioassays to screen for potential thyroid disrupting activity.

Overview of the Thyroid Endocrine System & thyroid disruption (Chapter 1)

In keeping with the first objective of this study, the thyroid endocrine system is reviewed, specifically addressing the morphology as well as the endocrinology of the thyroid system. A comparative approach is taken, to include current literature on the thyroid systems of non-mammalian vertebrate species. This basic primer to the thyroid endocrine system forms the foundation to understand the potential disruption of contaminants in the thyroid system. It will also aid the informed designing of screens and tests for thyroid toxicants since these assays must be developed based on what is known about thyroid endocrinology (Zoeller and Tan, 2007). The current state of knowledge about the known man-made and natural compounds that may affect the thyroid endocrine system is reviewed. From the literature, it was clear that a diverse range of compounds might disrupt or modulate the thyroid system. Reviews by Brucker-Davis (1998), Brown *et al.* (2004), Boas *et al.* (2006), Yamauchi and Ishihara (2006) and WHO show that environmentally relevant chemicals that may exert acute or chronic effects on the thyroid endocrine system include, polychlorinated hydrocarbons, polycyclic aromatic hydrocarbons, organochlorine pesticides, chlorinated paraffins, organophosphorous pesticides, carbamate pesticides, arsenic, cyanide compounds, methyl bromide, phenols, ammonia, metal, acid load, sex steroids, styrene compounds, perchlorate, halogenated Bisphenol A, Triclosan and several other pharmaceuticals. Although the list of potential thyroid endocrine disruptors is rather long, few studies have confirmed their biological impacts, for example, using *in vivo* exposure systems like XEMA or specific mode of action *in vitro* bioassays.

The comparative review approach showed that the inherent conservative nature of the thyroid system in vertebrates greatly contribute to the success of using non-mammalian species as model systems to assess potential TDCs. Although extrapolation to the human thyroid system may still be problematic, at least non-mammalian bio-indicators can be used as early warning systems.

Xenopus Metamorphosis Assay (XEMA) (Chapter 2).

The guidelines of OECD and USA-EPA for a semi-static exposure setup of the *Xenopus* metamorphosis assay (XEMA) used in the inter-laboratory validation studies coordinated by OECD (Kloas *et al.,* 2003; Degitz *et al.,* 2005; Opitz *et al.,* 2005; Tietge *et al.,* 2005) was followed to evaluate the potential to use XEMA locally. Tadpoles were bred in the laboratory and exposure to control chemicals started at the stage when the forelimbs emerged (premetamorphosis, NF-stage 48) (Nieuwkoop and Faber, 1956), for 21 days. The premise being that TH agonists will enhance development and TH antagonists will delay metamorphosis. Using developmental stage (NF) (Nieuwkoop and Faber, 1956), body size,

forelimb length and hindlimb length as endpoints the effects of control chemicals on the thyroid axis through the XEMA approach was assessed. Selected tadpoles from each exposure group were processed, in accordance with the XEMA guidelines prepared by the USA-EPA, for histological studies (OECD, 2007b; d). Histological inspection confirmed the results obtained by the USA-EPA. Although basic aspects of pathological conditions were observed, more material and study is needed to document histopathological conditions of the thyroid related to endocrine disruption. Following this exposure study the semi-static exposure and a simple flow-through system set-up was confirmed to suit low-budget laboratories that do not have the infrastructure and labour support of large laboratories. For example, triplicate exposure groups (n=30 tadpoles) were used and exposure water was changed every third day. Tadpoles were measured and weighed at the onset of the exposure. In the initial setup, tadpoles were removed and data recorded after a 14-day exposure period. On day-21 all tadpoles were sacrificed and measured. Five tadpoles per replicate group were removed, fixed and preserved for histological studies. Following exposure, apart from the staging of the development, morphometric measurements of the developing forelimb and hindlimb were also taken. Results indicated that the exposure was successful and compared well with the results obtained in the International inter-laboratory studies conducted by the OECD and USA-EPA. Thyroid hormone (T4) accelerated metamorphosis and PTU (propylthiouracil, inhibitor) inhibited progression of metamorphosis. We also include sodium perchlorate and Bisphenol A as potential toxicants. Tadpoles exposed to perchlorate showed severe arrest of metamorphosis while those exposed to Bisphenol A showed moderate inhibition of metamorphosis. Histopathological analyses confirmed cellular effects of control chemicals. In both inhibitors, PTU and sodium perchlorate, goiterous thyroids were observed and in the positive control group receiving thyroxine (T4) a decreased thyroid volume with limited colloidal area confirmed. No clear thyroid inhibition could be confirmed using the developmental staging, morphometrics or histopathology endpoints.

Selected Case studies (Chapter 2)

The *Xenopus* metamorphosis assay (XEMA) was applied to three case studies in which water samples collected from field sites were brought to the laboratory for exposure. Two of these studies were conducted as semi-static studies (Kuils-Eerste River system & Windhoek WWTP) and one as a flow-through study (Zandvliet WWTP). The Kuils-Eerste River exposure was the first attempt to collect large volume water samples from selected river sites for renewal every third day. The development of the exposed tadpoles was not dramatically affected although some indication was found of possible stimulation/ enhancement at sites. No histopathology analyses were done with these tadpoles. The

range of sites selected from the Municipality of Windhoek WWTP included source water, final treated water and water collected from a distribution point. When comparing the tadpoles exposed to the samples to the laboratory control group, no significant developmental effects were observed. In one sample, in the third week mortality increased and development slowed down. The affected group was exposed to water collected after the activated charcoal treatment point. In spite of the increase in mortality thyroid histopathology did not show clear thyroid disruption evidence. More research is needed to explain the incidence of high mortality and possible effects on the thyroid, however, it was concluded that the mortality in the last week of exposure might be linked to some unknown toxic effect. This study proved that large volumes of water could be transported over long distances to the laboratory for exposures over a three-week period. In the case of the Zandvliet WWTP study, a simple flow-through setup was devised using an 18-channel peristaltic pump. For this preliminary study, the final effluent was used either diluted (50%) or undiluted (100%). No significant developmental effects were found in any of the exposure groups and exposure to undiluted samples did not result in any mortalities. Although in the individual tadpoles exposed to undiluted treated effluent (100%) some indication of thyroid inhibition could be found, no significant increase in the thyroid or colloidal area were evident and histopathological indicators were unconvincing. This result seemed surprising since a recent study showed relatively high concentrations of phenols and phthalates (Olujimi *et al.,* 2012) known to inhibit thyroid activity (Ishihara *et al.,* 2003; Shen *et al.,* 2011). However, it seems that the concentrations of the TDCs (phenols and phthalates) in the final effluent were relatively low when compared with the exposure concentrations mentioned in Shen *et al.* (2011). Although the flow-through system resulted in large quantities of wastewater it was less labour intensive.

From the selected environmental studies used in this study, it is clear that in South Africa the application of the XEMA screening tool is possible, given that the transport (overnight courier transport) of relatively large volumes of water can be undertaken and large volumes of wastewater can be discarded/treated on a daily basis. Special effort will be needed for treatment (activated charcoal filtration) of flow-through effluent when conducting chemical studies with high nominal concentrations of the chemical (or mixture of chemicals) included in a concentration series.

Short-term exposure studies: Molecular markers (Thyroid receptor (TR) mRNA expression) (Chapter 3).

In this study we selected two sentinel species, the African Clawed frog, *Xenopus laevis*, endemic to Southern Africa, easy to breed in captivity, maintain in exposure studies and studied on a global scale, and the Mozambique tilapia (freshwater fish), *Oreochromis*

mossambicus, widely used as an aquaculture species and occurring naturally several Southern African rivers.

Xenopus Thyroid Receptor (TR) gene expression:- Although XEMA offers a whole-body exposure approach, it remains a rather long exposure (21 days) and the search for shorter-term exposures is always on. Molecular markers have been shown to be sensitive biomarkers showing fast response to chemicals binding to hormone receptors. The pre-metamorphic *Xenopus laevis* tadpole displays competence to respond to exogenous TH by upregulation of the TH responsive gene expression through the upregulation (increased mRNA expression) of thyroid receptor-beta (TRβ) protein (Shi, 1999). Thyroid hormone (TH) acts primarily via nuclear receptors (TRs). Two isoforms, TRα and TRβ, are known for most vertebrates. TRβ isoform is generally associated with TH responses in target tissues. The upregulation of the TRβ gene by TH is unique and occurs within 48 hours after T3 exposure (Crump *et al.,* 2002; Zhang *et al.,* 2006; Opitz and Kloas, 2010). TRβ gene expression is generally low during the pre-metamorphosis stages (NF stage 50-51) (Shi, 1999), but highly responsive to T4 and T3 hormones. The protocols previously described (Veldhoen and Helbing, 2001; Crump *et al.,* 2002) were followed and NF stage 54 *Xenopus laevis* tadpoles were exposed to thyroid hormone (T3) and Bisphenol A (suggested to be a TH antagonist (Moriyama *et al.,* 2002; Zoeller *et al.,* 2005) as well as to the combination of T3 and Bisphenol A (to assess antagonism or synergism) for 48 hours. The utility of using real-time quantitative PCR (RT-QPCR) methodology (Opitz *et al.,* 2006b; Zhang *et al.,* 2006) to quantify the expression of the TRβ gene following a 24-hour T3 exposure was confirmed. Although, no significant upregulation of the TRβ mRNA after exposure to Bisphenol A was found, upregulation of TRβ mRNA occurred following exposure to T3 in mixture with Bisphenol A. The potential of using QPCR methodology to TRβ mRNA after a short-term exposure (24-48 hours) was confirmed and could be used in combination with other *in vivo* exposures, like the *Xenopus* metamorphosis assay (XEMA).

Oreochromis mossambicus Thyroid Receptor (TR) gene expression:- The success of using short-term exposures and quantifying the TRβ mRNA in *Xenopus laevis* tadpoles was the impetus for characterizing similar genes in a local freshwater fish species, *Oreochromis mossambicus*. This species has a wide distribution in natural river systems in South Africa and is widely used in ecotoxicological studies (laboratory and field studies). Moreover, it is a well-known aquaculture species and the availability of juvenile and adult fish allows for exposure designs including large numbers of same stage fish in a laboratory exposure set-up. In this study, total RNA was extracted and TRβ mRNA characterized resulting in the design of specific *Oreochromis mossambicus* TR primers. PCR primer sequences for TRα and TRβ cDNA in *O. mossambicus* were initially designed from the closely related *Oryzias*

latipes. Amplified DNA fragments were cloned into vectors and transformed into *E. coli* to sequence and confirm the amplicons. For the first time, relative quantifications of gene expression of *O. mossambicus* TRβ and TRα can now be performed. In the second part of this study, natural expression of TR mRNAs was screened for in various tissues followed by an exposure experiment (48 hours) using juvenile fish (20 dpf). Expression in the ovarian tissue and liver tissue was found to be higher than in the other tissues sampled in the juvenile fish. Subsequent expression was therefore quantified in either ovarian of liver tissues. Control exposure included thyroid hormone (T3) and two environmental chemicals, Bisphenol A (component of plastics) and Endosulfan (pesticide) as well as exposure to combinations of the chemicals and T3. This study confirmed the upregulation of thyroid hormone receptors after a 48-hour exposure of juveniles (20 dpf) to thyroid hormone (T3). Bisphenol A exposure did result in the upregulation of TRβ showing some indication of synergism when exposed in combination with T3. Endosulfan on the other hand did not result in the up-regulation of TRβ but when exposed in combination with T3 a significant decrease in TRβ expression was evident. Similar to *Xenopus laevis* tadpoles, juvenile tilapia fish hold great potential to be used in short-term exposure studies to indirectly assess effects on the thyroid system by evaluating the differential expression of the TRβ mRNA.

Both the *Xenopus laevis* tadpole (pre-metamorphic) and *Oreochromis mossambicus* juvenile (20 dpf) short-term exposure studies confirmed that TRβ expression responded to changes in exogenous TH and that this response can be influenced by the presence of exogenous environmental chemicals, like Bisphenol A (agonistic) and the pesticide, Endosulfan (antagonistic). This study corroborate the suggestion of Opitz *et al.* (2006) that short-term bioassay involving RT-QPCR analysis of TRβ expression following single or combined treatments of pre-metamorphic tadpoles or juvenile fish with T3 and suspected TDCs, represents a promising additional screening approach when screening for thyroid disrupting activity.

Available in vitro screening tests for potential thyroid disruption (Chapter 4)

A literature review of the diversity of laboratory-based tests was included and clearly, the battery of *in vitro* tests is growing fast, most of these addressing specific mechanisms of action. Generally, *in vitro* assays are not as labour intensive and of shorter duration when compared to *in vivo* assays, like for example the *Xenopus* metamorphosis assay (XEMA). However, these assays can mostly only be applied to investigate specific pathways/endpoints in the complex regulatory network associated with the thyroid endocrine system. Although the complexity of the thyroid system imply that a battery of tests will be impractical (Zoeller and Tan, 2007), the development and validation of screening assays for TH interaction or other endpoints in the thyroid system, lags behind development of similar

first tier bioassays developed for reproductive endocrine systems. Although the risk of false negatives is high when using *in vitro* assays to screen for thyroid disruption (a system with multiple points of interference), early warning signposts remains valuable before embarking on *in vivo* exposure experiments.

In summary, the following *in vitro* tests have been used in the past: The classical receptor binding assay for thyroid receptor binding, similar to the estrogen and androgen receptor binding assays, has been attempted (Date *et al.,* 2002). A rat pituitary tumur cell line (GH3 cells), has been used to detect generalized disruption of TR action (TScreen) analogous to the EScreen for estrogenicity/anti-estrogenicity. GH3 cells have both TRα and TRβ receptors and proliferate when exposed to T3 (Ghisari and Bonefeld-Jorgensen, 2005; Gutleb *et al.,* 2005; Schriks *et al.,* 2006; Zoeller and Tan, 2007).

Recently, a reporter gene assay, expressing luciferase under control of the TH receptors, TRα (Jugan *et al.,* 2007b) and TRβ (Sugiyama *et al.,* 2005) has been used. TSH-stimulated cAMP production in vitro, using Chinese hamster ovary cells transfected with the recombinant TSH receptor was used to study the effect of compounds on the disruption of TSH binding to its receptor (Santini *et al.,* 2003). Recently, a TRα and TRβ transient transactivational assay system using mammalian cells (HEK293) was established (Oka *et al.,* 2012). Another *in vitro* assay, aimed at a specific pathway, for example, FRTL-5 cells (Fisher rat thyroid line) have the ability to concentrate iodide (Zoeller and Tan, 2007b). A T4-transthyretin binding assay (TTR) was used to study disruption of the T4 transport in the blood by the plasma protein carrier, transthyretin (Lans *et al.,* 1994; Yamauchi *et al.,* 2003; Murata and Yamauchi, 2007).

Several studies also report on the use of *ex vivo Xenopus* tail tissue culture assays. In these assays the tails of NF-stage 52 to NF-stage 54 tadpoles are removed surgically and placed in culture. The tails are then exposed to different compounds including control hormones and measured every 24 hours. The variation in tail regression is then compared, the rationale being that TH induces regression of the tail and substances could delay or increase the regression process (Iwamuro *et al.,* 2003; Schriks *et al.,* 2006). Recently, Hornung *et al.* (2010) describe the use of an *in vitro (ex vivo*) bioassay using thyroid glands of *Xenopus laevis* tadpoles.

CONCLUSIONS

Reviewing the thyroid disruption literature base showed that a knowledge explosion is currently happening in this field. This is partly the result of the concern that thyroid disruption caused by contaminants in the environment, including the aquatic environment is

a major EDC threat. The development and validation of the *Xenopus* metamorphosis assay (XEMA) was an important development and although a long and laborious assay to conduct, it is a good assay to assess how the whole thyroid system response to a thyroid disrupting contaminant (of which the specific mechanism of action may still be unknown). Using a semi-static set-up, allows a reasonably simple exposure system to screen for potential thyroid disrupting activity (specifically TH mediated). Morphological and developmental endpoints are well developed. More research is needed to link histopathological changes to endocrine disrupting activity. The use of short-term TR expression bioassays, involving RT-QPCR analysis of TRβ mRNA, holds great potential as an indirect bioassay. Several *in vitro* bioassays are available and used only by a few researchers to address specific questions regarding certain modes-of-actions/endpoints in the complex thyroid endocrine system. Clearly, this field will rapidly expand in the near future.

All the aims were reached, except for the development of additional biochemical endpoints and the incorporation thereof into specific bioassays. Although research is needed in this regard, the inclusion of gene expression quantification paved the way for conducting short-term *in vivo* assays.

One of the benefits/outcomes of this project is the fact that it confirmed that we have the capacity in South Africa to do assessments for thyroid endocrine disruptors. The project will also benefit the managers and researchers since they gain background information in the potential of thyroid disruption by contaminants in our water resources. Since we succeeded in validating the XEMA assay we also present procedures to conduct the XEMA assay. In addition, this project for the first time also introduces the idea of extending EDC biomarkers to the molecular level. Valuable information on the potential application of quantifying specific gene expression and the effects of aquatic contaminants on these genes is presented. It is therefore clear that at several levels and in several areas related to endocrine disruption in South Africa, this project has expanded capacity and should the screening and testing for thyroid disruption be initiated on a national scale, we now have the capacity to implement XEMA and short-term molecular assays.

RECOMMENDATIONS FOR FUTURE RESEARCH

- Screening assays for thyroid disruption should, as a matter of urgency, be used more widely, at least in water treatment laboratories.

- Training workshops should take place to train researchers to use the XEMA testing approach, including the in house breeding of breeding *Xenopus laevis* tadpoles.

- Training workshops in data analyses and thyroid histopathology should be held.

- Research towards refining the XEMA testing by reducing the period of exposure and conducting the exposure in the early pro-metamorphosis phase of development.

- Research on the set-up, development and validation of *in vitro* assays related to the most important points of disruption should continue.

- Expand the molecular endpoints (mRNAs) and use of RT-QPCR in short-term exposures of tadpoles.

- Research and establish the epidemiology of thyroid related diseases in South Africa that specifically link to environmental contaminants.

- Future research should target and assess human activities known to produce compounds that potentially may disrupt the thyroid systems of wildlife and humans.

- Initiate research to develop basic and ecological risk assessment models that specifically include thyroid disruption as an important risk factor.

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Mrs APM Moolman	Water Research Commission (past Chairperson)
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5‘DII	5‘deiodonase type II enzyme
5’DI	5‘deiodonase type I enzyme
5D	5-deiodonase type III enzyme
Alb	Albumin plasma protein
AMA	Amphibian metamorphosis assay
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AquaStel	A registered South African company with links to the Division of Aquaculture at Stellenbosch University
BPA (BisA)	Bisphenol A
BTEB	Basic transcription element binding protein
C-cells	Parafollicular cells
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CHO	Chinese hamster ovary cells
CLO_4^-	perchlorate
CRH	Corticotropin-releasing hormone
Ct	Cycle threshold value generated in real-time quantitative PCR
Cyp	Cytochrome P450 enzymes
DDT	1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane
DEPC	Diethyl pyrocarbonate

DI	Deiodinase enzyme
DIT	Diiodothyrosine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease, an enzyme that catalizes cleavage in DNA
DO	Dissolved Oxygen
dpf	Days past fertilization
DRP	Detailed draft paper
E-screen	The *E-screen* assay, is a bioassay based on the ability of MCF7 (human breast cancer cell-line) cells to proliferate in the presence of *estrogens*
E. coli	Escherichia coli bacteria
EC50	Effective concentration in 50% of exposed animals/cases
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee (USA-EPA)
EtOH	Ethyl Alcohol
FRTL-5	A rat thyroid cell line
GAC	Granular activated charcoal
GH	Growth hormone
GR3	A rat pituitary tumor cell line
GRIP1	A translational co-activator of thyroid and retinoic receptors
GWRC	Global Water Research Coalition
H&E	Hematoxylin & Eosin staining
hCG	Human chorionic gonadotrophin
HEK293	Mammalian cell line

HPT	Hypothalamus-pituitary-thyroid
Hr(s)	Hour(s)
I^-	Inorganic iodide
I.U.	International units
L	Liter, 1000 mL
Luc	luciferase
mg	Milligram
MIT	Mono-iodothyrosine
mL	Milliliter
mRNA	Messenger RNA
MS-222	Tricaine methanesulfonate
MTI	Antithyroid agent, Methimazole
NA/K – ATPase	Sodium-potassium adenosine triphosphatase enzyme
NF	Nieukoop & Faber (1956)
NIS	Sodium (Na^+)-Iodide (I^-) symporter
nM	Nano-Mol
NO_3^-	nitrate
OECD	Organization for Economic Cooperation and Development
PC12	Mammalian cells
PCBs	polychlorinated biphenyls
PCR	Polymerase chain reaction
PF	Post fertilization

PRL	Prolactin
PTU	Propylthiouracil
qRT-PCR	Quantitative Reverse-transcription Polymerase Chain Reaction
RAI	Radioactive iodide
RAIU	Radioactive iodide uptake
REC10	10% relative effective concentration
RNA	Ribonucleic acid
RNase	Ribonuclease, catalyses the degradation of RNA
RO	Reverse osmosis
RT-PCR	Real-time reverse-transcription Polymerase Chain Reaction
rT3	3,3',5'-Teriiodothyronine
RXR	Retinoid X receptor, 9-cis-retinoic acid receptor
SANS	South African National Standards document
SASS	South African Scoring System
SCN^-	Thiocyanate
SD	Standard deviation
SOP	Standard operating procedure
SP (sPER)	Sodium perchlorate
STW	Sewage treatment works
SVL	Snout-to-vent-length
SYBRgreen	A cyanine (fluorescent) dye used as a nucleic stain in molecular biology
T-screen	An *in vitro* thyroid hormone bioassay based on the proliferation of rat pituitary tumor cells (GH3)

T2	Di-iodo-thyronine, thyroid hormone
T3	3,5,3’-Triiodothyronine, thyroid hormone
T4	Thyroxine, 3,5,3’,5’-Tetraiodothyronine, thyroid hormone
TBG	Thyroxine binding globulin
TBL	Total body length
TDC(s)	Thyroid-disrupting compound (contaminant)
TG	Thyroglobulin
TH	Thyroid hormone
THBP	TH binding protein
TIF-2	Transcriptional intermediary factor 2
TL	Total length
TPO	Thyroid peroxidase enzyme
TR	Thyroid receptor
TRE	Thyroid response element
TRH	Thyrotropin-releasing hormone
TRα	Thyroid receptor-alpha
TRβ	Thyroid receptor-beta
TSH	Thyrotropin, Thyroid-stimulating Hormone
TSH-r	Thyrotropin- , Thyroid-Stimulating Hormone receptor
TTF-1	Thyroid transcription factor -1
TTR	Transthyretin
UK	United Kingdom
USA	United States of America

USA-EPA	United States of America Environmental Protection Agency
UV	Ultra violet light
WHO	World Health Organization
WQ	Water quality
WWTP	Waste water treatment plant
X. laevis	*Xenopus* laevis
XEMA	*Xenopus* metamorphosis assay
XL58	*Xenopus laevis* cells
xTR	*Xenopus laevis* thyroid receptor
xTTR	*Xenopus laevis* transthyretin

1 CHAPTER 1: INTRODUCTION AND OBJECTIVES

Global concerns have been raised about the possibility that environmental chemicals (mostly man-made) may interfere with the endocrine systems of wildlife and humans (Damstra *et al.,* 2002; OECD, 2006; Zoeller *et al.,* 2012; Bergman *et al.,* 2013; Heindel *et al.,* 2013b). These concerns primarily stem from an extensive knowledge base reporting evidence that wildlife, including invertebrates, examples from all vertebrates groups, fish, amphibians, birds and mammals, as well as ecosystem-health in general, may be compromised by endocrine disruptive activities of water-borne man-made chemicals. In addition, increased endocrine related diseases in humans further contribute to this concern (Colborn, 2004; Brenner and Galvez, 2007; Filby *et al.,* 2007; Trudeau and Tyler, 2007; Woodruff, 2007; Heindel *et al.,* 2013b). Laboratory experimentation and field studies related reproductive health of wildlife and humans contributed to the formulation of the so-called endocrine disruption hypothesis (Krimsky, 2000; Damstra *et al.,* 2002; Gore, 2007; Diamanti-Kandarakis *et al.,* 2009; Heindel *et al.,* 2013b). Concerns mostly focus on the possible adverse effects on the developmental and reproductive biology of organisms inhabiting the aquatic environment, as well as humans as major end-users of water resources. Endocrine disruption of the control and functioning of the reproductive system by far attracted the most attention (Clark *et al.,* 1998; Bogi *et al.,* 2003; Bergman *et al.,* 2013; Heindel *et al.,* 2013a). However, several lines of evidence suggest that environmental chemicals may also interfere/modulate with the normal functioning of the thyroid endocrine system (Brucker-Davis, 1998; DeVito *et al.,* 1999; Leatherland, 1999; Jahnke *et al.,* 2004; Brent *et al.,* 2007; Tan and Zoeller, 2007; Kloas *et al.,* 2009; Jobling *et al.,* 2013).

The thyroid hormones are important for normal growth and development in all vertebrates (fish, amphibians, birds and mammals)(Zoeller and Rovet, 2004; Norris and Carr, 2006; OECD, 2006; Jobling *et al.,* 2013). In addition, thyroid hormones may influence the activity of a wider variety of tissues and biological functions than do any other hormone, for example osmoregulation, metabolism, and post-hatch metamorphosis in fish and amphibians (Norris and Carr, 2006; Blanton and Specker, 2007; Jobling *et al.,* 2013). These iodine-containing hormones are derived from the amino-acid, tyrosine (Norris and Carr, 2006). Tetraiodothyronine (T4, thyroxine) has iodine molecules attached to four carbons while triiodothyronine (T3) lacks an iodine. The regulation of thyroid hormone release and delivery to tissues and cells during development and in the adult, represents a complex system, including an extended network of feedback systems (Norris and Carr, 2006; OECD, 2006; Fort *et al.,* 2007; Zoeller *et al.,* 2007a; Jobling *et al.,* 2013). Thyroid hormone (TH) synthesis is controlled by thyrothropin (TSH) released from the pituitary gland as part of the Hypothalamus-Pituitary-Thyroid axis (HPT). The knowledge base on environmental

contaminants acting/interfering at several sites within the HPT, including the effects of environmental iodide deficiencies or interference with the uptake of iodide recently increased (Brucker-Davis, 1998; OECD, 2006; Zoeller *et al.,* 2007a; Kidd *et al.,* 2013).

Given the importance of thyroid hormone for growth and development and several physiological functions in vertebrates, including humans, it is seems reasonable to be concerned that environmental contaminants could influence thyroid functioning and signalling to the extent that populations would be adversely affected (Brucker-Davis, 1998; Hutchinson *et al.,* 2006; Miller *et al.,* 2009). Because of the highly conserved nature of TH life history (synthesis, transport, regulation, and metabolism) environmental contaminants that affect thyroid function in one vertebrate species may well affect the TH functioning in an other, including humans (Cheek *et al.,* 1999; Norris and Carr, 2006). Zoeller and Tan (2007a) recognized that while the general functionality of the thyroid system may be the same among vertebrates, there are species-specific differences to be considered; for example, the role of thyroid hormones in controlling amphibian metamorphosis is likely to be different in human development. However, applying vertebrate models to answer the question of whether, or how a contaminant interferes with, or modulates thyroid hormone action or any other aspect of the life history of these hormones, does contribute in initial screening programmes.

Considering the important and wide-spread role of thyroid hormones, and the concern that thyroid disruption may be more than a hypothesis, the development and validation of bioassays to identify potential thyroid-disrupting contaminants (TDCs) received much attention recently (DeVito *et al.,* 1999; Degitz *et al.,* 2005; Opitz *et al.,* 2005; OECD, 2006; Fort *et al.,* 2007; Zoeller and Tan, 2007a). The aim is to capture the variety of potential points of modulation within the thyroid endocrine system that may be disrupted by exposure to thyroid-disrupting contaminants (TDCs). The one outstanding feature of the thyroid system is the relative large number of regulatory points, which makes it difficult to put together a battery of *in vitro* screens. It is therefore not surprising that the initial focus in screening for general thyroidal functional effects was on *in vivo*, for example the *Xenopus* metamorphosis assay (XEMA) (Fort *et al.,* 1999b; Norris and Carr, 2006; OECD, 2006; Kloas *et al.,* 2009), rather than *in vitro* bioassays. Assays like XEMA however are labour intensive and may include relatively long exposure periods (Degitz *et al.,* 2005; Opitz *et al.,* 2005; Miyata and Ose, 2012). Molecular endpoints (TH dependent gene expression), for example thyroid hormone receptor (TR) mRNA expression (TRα and TRβ) have been quantified following short-term exposure (24 to 48 hours)(Crump *et al.,* 2002a; Opitz *et al.,* 2006a; Zhang *et al.,* 2006a; Helbing *et al.,* 2007b). Recently, specific *in vitro* assays were

incorporated in testing alongside *in vivo* exposures to address hypotheses regarding specific modes-of-action (Gutleb *et al.,* 2005; Schriks *et al.,* 2006).

Both the Organization for Economic Cooperation and Development (OECD) and the USA-EPA established endocrine disruptor testing and assessment working groups with the mandate to produce detailed review papers (DRPs) and subsequently develop and validate batteries of bioassays for EDC testing (Ankley *et al.,* 1998; EDSTAC, 1998; Fenner-Crisp *et al.,* 2000; Kanno *et al.,* 2003; OECD, 2006; Tan *et al.,* 2007; Tan and Zoeller, 2007). Both organizations published extensive DRPs and the USA-EPA's EDSP (Endocrine Disruptor Screening Programme) is currently validating assays and have recently submitted several first tier assays for external peer review (OECD, 2008b; 2009).

Because of the pivotal regulatory role of thyroid hormone (TH) during amphibian metamorphosis and because of extensive research that has been done at several levels of the biological organization (Shi, 1999; Kloas and Lutz, 2006; Kloas *et al.,* 2009), amphibian metamorphosis has been targeted as an *in vivo* model system to screen for thyroid disrupting activity (Touart, 2002; OECD, 2006; Fort *et al.,* 2007). The USA-EPA's DRP reviewed candidate anuran species and recommended that the African Clawed Frog, *Xenopus laevis* be used in a metamorphosis assay (*Xenopus* metamorphosis assay, XEMA) (Touart, 2002; Degitz *et al.,* 2005; Opitz *et al.,* 2005; Tietge *et al.,* 2005a; Fort *et al.,* 2007). The XEMA is a morphological assay designed to detect effects of contaminants on metamorphic development, the rationale being that morphological alterations during development reflect effects on TH function. Although the conceptual framework for the XEMA has been developed and the assay recently validated in the USA, Germany, UK and Japan the need for validation here locally, exist, since scientists from South Africa, as a non-OECD member, did not have the means or opportunity to participate in the ring-testing programme coordinated by OECD and USA-EPA to validate XEMA. Moreover, it has been recognized that there is a need to investigate additional endpoints as biomarkers for thyroid disruption to be implemented alongside XEMA or as short-term preliminary screening tools. For example, additional biochemical markers for specific pathways as well as molecular tools (differential gene expression of TH receptors) may be used as early warning sign-posts for potential thyroid disruption. There is also a need to study histological (pathological) endpoints in more detail. Although the original XEMA was set-up as a semi-static renewal exposure system, the recommended protocol for XEMA include a sophisticated flow-through system (Degitz *et al.,* 2005; OECD, 2009). The question is, is the semi-static renewal system (Kloas *et al.,* 2003) acceptable for use in developing countries and is there simple flow-through systems that could work for screening environmental water?

Apart from using amphibians as models for thyroid disruption screening, the use of developing fish has also been recognized (Crane *et al.,* 2004; 2005; 2006; Blanton and Specker, 2007). Apart from *Xenopus laevis*, several local fish species have the potential to be employed as bioindicator models in EDC studies, such as, *Oreochromis mossambicus*, a species extensively used in the aquaculture industry (Esterhuyse *et al.,* 2008b; Esterhuyse *et al.,* 2009; Mlambo *et al.,* 2009; Barnhoorn *et al.,* 2010; Esterhuyse *et al.,* 2010; Swart *et al.,* 2010) and the sharp tooth catfish, *Clarias gariepinus* (Barnhoorn *et al.,* 2004; Marchand *et al.,* 2008; McClusky *et al.,* 2008).

Although South Africa is part of the developing world, it has all the characteristics and human activities associated with a modern developed country. The potential for endocrine disruption is similar to countries in Europe and the Americas. However endocrine disruptor research activity remains limited with a low priority status, compared to developed countries. Initially, the focus was on studying the endocrine disrupting activity (mostly estrogenic activity, feminization) of contaminants like nonylphenol (De Jager *et al.,* 1999) and DDT (Dalvie *et al.,* 2004; Bornman *et al.,* 2007a; de Jager *et al.,* 2009; Van Dyk *et al.,* 2010; Bouwman *et al.,* 2011; Bouwman *et al.,* 2012; Gyalpo *et al.,* 2012). Since the realization that EDCs in South Africa may be a real environmental and human health concern, focus shifted to bioassay development and validation, which culminated in the establishment of a toolbox programme (De Jager, 2010). Initially, *in vivo* models were used to assess estrogenicity (Hurter, 2003; van Wyk *et al.,* 2005; Esterhuyse, 2008; Swart, 2008) and anti-androgenicity (van Wyk *et al.,* 2003), the shift the *in vitro* bioassays followed (Swart and Pool, 2009b; a; De Jager, 2010; Swart *et al.,* 2010). Although in recent years few studies directly investigated EDC activity in aquatic systems most of these were focused on reproductive disruption (van Wyk *et al.,* 2005; Bornman *et al.,* 2007b; Barnhoorn *et al.,* 2009). The thyroid disruption programme, developed from the initial mandate to investigate the endocrine disruptor potential of all man-made chemicals, was initiated the USA-EPA and OECD, with the initial focus on the thyroid controlled metamorphosis phenomenon using a South African aquatic frog species, *Xenopus laevis* as model organism (OECD, 2009). Despite the South African origin of *Xenopus laevis*, South African scientists did not have the opportunity to participate in the development and subsequent ring testing of the "*Xenopus* metamorphosis assay, XEMA" to screen for thyroid disrupting contaminants (TDCs) or activity in environmental waters. Through the subsequent explosion of research around the world concerning potential thyroid disruption and the development and validation of the XEMA protocol the gap and need in the local EDC testing capacity is recognized. Apart from laboratory testing for TDC activity of selected chemicals, the use of XEMA or related short-term molecular assays testing environmental waters is made possible locally since *Xenopus laevis* occur

naturally in our aquatic environments. With the knowledge base regarding EDC effects of an aquatic amphibian species like *Xenopus laevis* as well as local freshwater fish models like *Oreochromis mossambicus* growing, the potential to eventually understanding the health and ecological risk (including wildlife and humans) of EDCs in South Africa becomes a real possibility. The overall objective of this project was therefore to setup and evaluate the XEMA approach (OECD, 2009) to identify thyroid disruption activity, evaluate the potential to actually screen environmental water using *Xenopus laevis* tadpoles. At the same time the potential to include freshwater fish species as models (at least in short-term molecular studies) was also investigated.

Objectives

In light of the need establish an EDC screening and testing programme in South Africa to assess and manage EDCs in our water resources, the following aims, specifically addressing the thyroid disruption issue, were formulated for this study:

AIM 1:

To introduce the thyroid endocrine system, concepts of thyroid disruption, summarize and convey the current knowledge regarding and the potential disruption of this system.

AIM 2

To review, set-up and validate the set-ups regarding the *Xenopus* Metamorphosis Assay (XEMA) (OECD/USAEPA protocol) for screening and testing water-borne contaminants for thyroid disrupting activity.

AIM 3

To investigate the different aquatic exposure systems: semi-static exposure vs. flow-through exposure.

AIM 4

To describe potential endpoints for thyroid disruption, morphological, histological, molecular (gene) and other biochemical endpoints.

AIM 5

To report on the potential short-term *in vitro* bioassays to screen for thyroid disruption.

2 CHAPTER 2: BACKGROUND

2.1 General background about Hypothalmus-Pituitary – Thyroid (HPT) axis

In this section an outline of the HPT axis, with brief descriptions of the different functional levels of the axis will be given. Reviewing the thyroid system starts from a mammalian perspective but it is well-recognized that the thyroid system is highly conserved among vertebrate species (Norris and Carr, 2006). The histology of the thyroid and structure of the thyroid hormones are similar to that of their mammalian counterparts (Norris and Carr, 2006). The implication being that the information on thyroid hormone production and control of secretion, including feedback through the hypothalamic-pituitary-thyroid (HPT) axis broadly will apply for all vertebrates. Since the acceptance of the potential of endocrine disruptors to have effects beyond the reproductive system also including the thyroid systems, several excellent reviews, broadly vertebrate and group-specific, explaining all aspects of the thyroid system have been published (Norris and Carr, 2006; OECD, 2006; Fort *et al.,* 2007; Zoeller *et al.,* 2007b; Denver *et al.,* 2009; Kloas *et al.,* 2009; Zoeller, 2010; Gilbert *et al.,* 2012; WHO, 2013). Following the basic background summary, the potential disruption of HPT axis, but specifically the thyroid endocrine system will be reviewed (WHO, 2013).

The control of the thyroid gland happens through the HPT axis. Specific neurons (cell bodies) located in the hypothalamus (paraventricular nucleus) synthesize thyrotropin-releasing hormone (TRH). Thyrotropin-releasing hormone is the main physiological stimulator of thyroid stimulating hormone (TSH) also referred to as “thyrotropin”, a glycoprotein hormone produced by the thyrotroph cells located in the anterior pituitary gland in mammals (Kopp, 2001; Norris and Carr, 2006; OECD, 2006; Fort *et al.,* 2007). Corticotropin-releasing factor (CRF) is suggested to be the primary hypophysiotropin for TSH in amphibians (Denver, 1998; Fort *et al.,* 2007). Although TRH-producing neurons are widely distributed in the brain, several have their axon-endings on the capillary networks in the median eminence, a neurohemal area of the hypothalamus. These capillary networks form the hypothalamic-pituitary-portal system, the principal blood supply to the anterior pituitary gland.

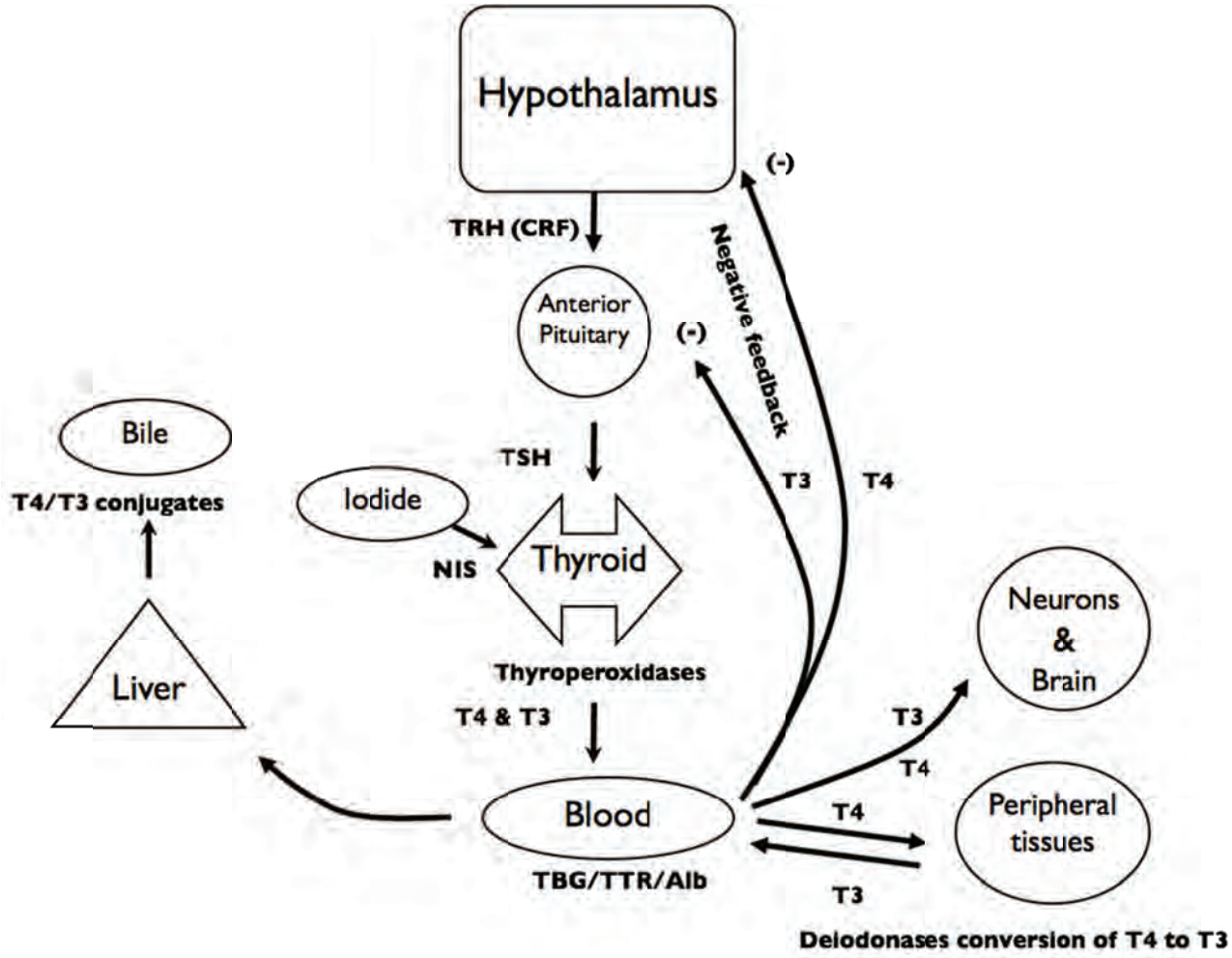

Figure 1: Simple diagram of the hypothalamus-pituitary-thyroid axis (HPT) . TSH, Thyroid stimulating hormone. TRH, thyrotropin releasing hormone. CRF, Corticotropin-releasing factor. T4, tetra-iodothyronine (thyroxine). T3, triodothyronine. TGB, thyroxine-binding globulin. TTR, transthyretin. TBG, Thyroxine binding globulin. Alb, albumin. NIS, Sodium-Iodide symporter. The information in the figure are based on diagrams presented in several recent reviews (Boas *et al.,* 2006; OECD, 2006; Crofton, 2008; Jobling *et al.,* 2013)

When neurosecretory neurons (TRH-producing) are stimulated to secrete the releasing hormone, TRH is discharged into the hypophyseal portal system. Through this circulation, TRH is delivered to the anterior pituitary gland. Pituitary TSH is a glycoprotein hormone and is composed of an alpha (α) and beta (β) subunit (Wondisford, 1996; Abel *et al.,* 2001). Trophic hormones like TSH are released from the pituitary in response to the stimulation from the releasing hormones (TRH/CRH) secreted by the hypothalamus. External environmental factors as well as inputs from higher centers influence hypothalamic activity (OECD, 2006). Pituitary TSH binds to specific receptors on the surface of thyroid follicle cells, and communicates with target cells via the adenylate cyclase second messenger system. Through this avenue TSH increase the uptake of iodide into the follicular cells of the

thyroid, iodination of thyroglobulin (TG), synthesis and oxidation of thyroglobulin, uptake of TG from thyroid colloid and the synthesis of the thyroid hormones, tetra-iodothyronine (thyroxine)(T4) and triodothyronine (T3). The former being the major product released from the thyroid gland (Norris and Carr, 2006; OECD, 2006). Both T4 and T3 are iodinated derivates of the amino acid tyrosine (coupling of the two phenolic rings of two iodinated tyrosine). Thyroxine (T4) contains four iodine atoms on the thyronine ring structure whereas T3 has only three iodine atoms (see below).

2.2 General background on the Thyroid Gland and Thyroid hormone action

2.2.1 Thyroid gland

The mammalian thyroid gland consists of two lobes, one on each side of the trachea and attached to the trachea by connective tissue. A thin band of thyroid tissue (isthmus) crossing the trachea connects the two lobes. These lobes are richly vascularized and consist of fluid-filled spherical follicles (soccer ball-shaped structures, lined with epithelial cells). The hollow cavity (lumen) of each follicle is filled with a protein-rich fluid called colloid. The colloid is mainly composed of one large protein, thyroglobulin, the storage form of thyroid hormones. The colloidal area is surrounded by a single layer of epithelial cells (thyrocytes) (cuboidal to columnar shaped). These cells are tightly bound together by tight junctions on the lateral surfaces close to the apical (free) sides of the cells. The apical surfaces of the thyrocytes are characterized by the presence of numerous microvilli (outfoldings), a feature common to most absorptive epithelia (Norris and Carr, 2006). The basal membranes of the thyrocytes are close to the richly vascularised interfollicular connective tissue (stroma). Thyrocyte size (height of epithelium), colloid amount and staining quality are generally believed to vary along with secretory activity or degree of TSH stimulation. These features are therefore used in the histological assessment of thyroid function.

In general, the thyroid gland is structurally conservative among vertebrates (Norris and Carr, 2006; OECD, 2006). Mostly the gland is a collection of follicles as described for mammals, highly vascularized and encapsulated by connective tissue, although in most fish the non-encapsulated follicles may be distributed (scattered) in the pharyngeal region surrounding the ventral aorta, but and/or be found in other organs (Eales *et al.,* 1997; Norris and Carr, 2006; Geven *et al.,* 2007; Kloas *et al.,* 2009).

In mammals the thyroid gland also contains cells that secrete the hormone, calcitonin, involved in the control of calcium metabolism. These cells are found in the interfollicular tissue of the thyroid and referred to as parafollicular cells (C-cells). However, non-

mammalian thyroid gland does not have parafollicular cells and calcitonin-producing cells are mostly associated with the ultimobranchial gland (Norris and Carr, 2006).

2.2.2 Thyroid hormones

Thyroid hormones are formed through chemical modification of thyrosine residues in the peptide structure of thyroglobulin as it is secreted into the lumen of the follicle. The stored thyroglobulin, therefore provides a large reservoir of stored thyroid hormones for later processing and secretion by the thyrocytes (Rhoades and Tanner, 1995). Thyroglobulin (Tg)(conserved among vertebrates) is synthesized in the thyrocytes and transported (by exocytosis) to the colloid. It is iodinated at the interface of the apical cell surface and the colloid. The iodination of thyroglobulin is catalyzed by a thyroid peroxidase enzyme, bound to the apical membrane of the thyrocytes. Initially the product, mono-iodothyrosine (MIT) is formed and following a second iodination, diiodothyrosine (DIT) is formed. These iodinated residues, close to each other in the thyroglobulin precursor, then undergo a coupling reaction, forming the iodothyronine structure. In the case of coupling of two DIT residues, T4 is formed. Although T3, is also formed in this way (MIT + DIT) most of the thyroid hormone produced by the thyroid will be in the form of T4.

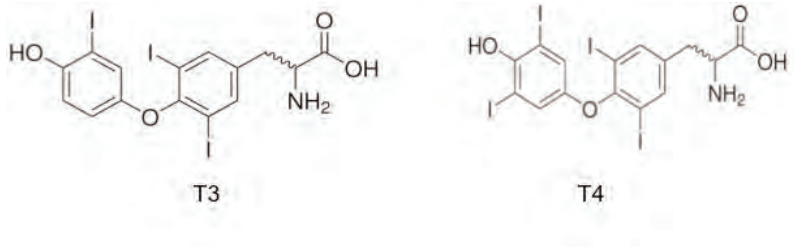

Figure 2. Structure of the two thyroid hormones, tetra-iodothyronine (T4,)(thyroxine) and triodothyronine (T3). Both hormones are synthesized in the thyroid gland. T4 is the precursor for T3 and is converted in the thyroid and peripheral target tissues to T3 by 5' deiodinases (see Figure 3)(Shi, 1999).

Iodide used for the iodination of thyroglobulin comes from the blood, through the capillary wall in close contact to the basal membrane of the thyrocyte (Figure 3). This uptake is facilitated by a sodium-dependent iodide co-transporter system (NIS) driven by a sodium gradient established by NA/K – ATPase pumps. In this way, follicular cells extract iodide from the blood and concentrate (20 to 40 fold) it in follicular cells. The activity of the NIS is controlled by TSH. Iodide, the form of inorganic iodine that enters the cell, is oxidized by thyroid peroxidase (TPO) before transferred to thyroglobulin (Figure 3). Since iodine is also required for a wider range of thyroid dependent functions and TSH serum levels

remains relatively normal despite fluctuations in daily iodine intake suggest that iodine has an autoregulatory role as well (OECD, 2006).

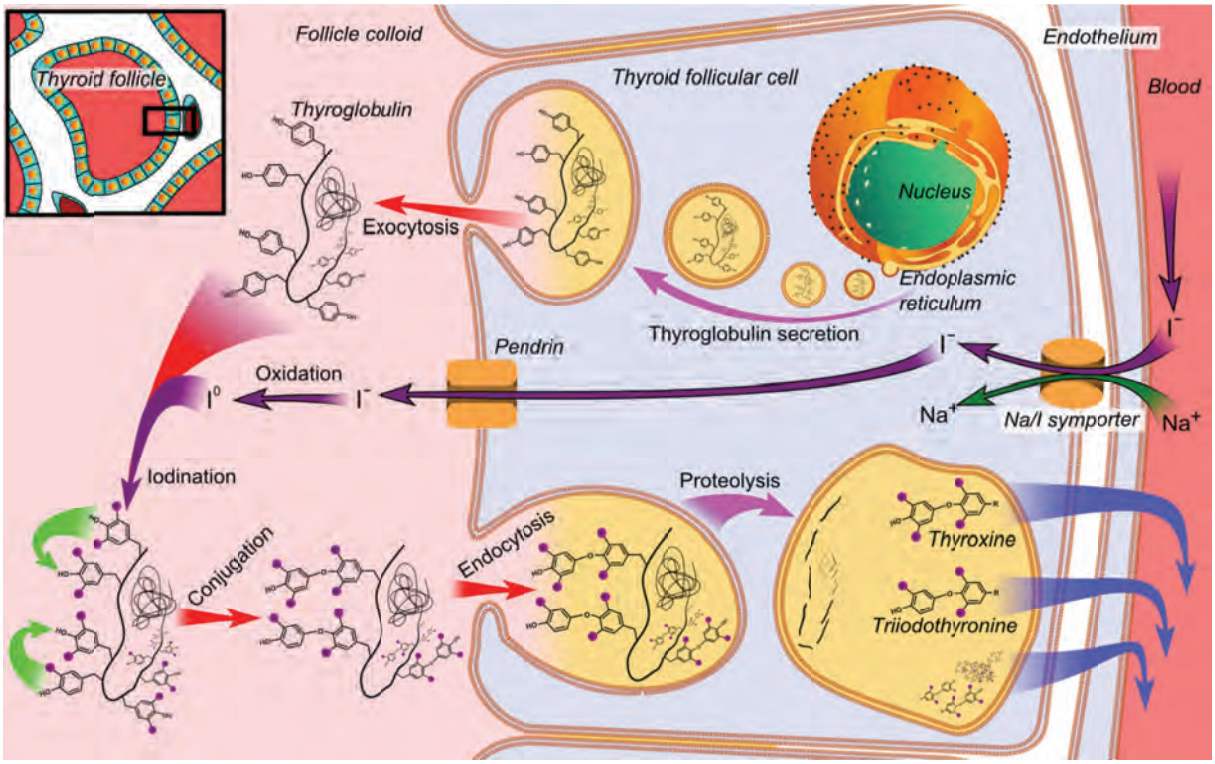

Figure 3. Thyroid gland: follicle cell showing the pathway of thyroid hormone synthesis (see text for detail). Diagram drawn and published online (Mikael Häggström in Wikipedia.org) without copyright. Accessed on 28 May 2013.

2.2.3 Thyroid hormone secretion

On TSH stimulation to secrete thyroid hormones, pinocytosis occurs on the apical membranes of the thyrocytes. These endocytotic vesicles (colloid droplets) migrate towards the basal region of the thyrocytes. Lysosomes at the same time migrate towards the apical region and on the way fuse with the colloid droplet. The enzymes of the lysosomes proteolytically hydrolyze the thyroglobulin and T4 and T3 and other residues are released into the cytosol (Rhoades and Tanner, 1995; Norris and Carr, 2006; Fort *et al.,* 2007)(Figure 3). Eventually, T4 and T3 diffuse from the follicular cell into the surrounding capillary network. At the same time cytosolic enzymes (deiodinases) catalyze the conversion of MIT and DIT to tyrosine and iodide (Figure 3). Waste iodide is then being recycled in the production pathway for thyroglobulin synthesis. Although primary synthesis of T4 and T3 happens directly in the thyrocytes, some metabolic conservation of T4 to T3 happens in

peripheral target tissues. This transformation is normally catalyzed by a 5'-deiodinase enzyme. Most of the physiological effects of thyroid hormones can be attributed to T3. Some of the T3 formed in the thyrocytes before leaving the thyroid gland, approximately 40% of T4 is converted in the liver to T3, therefore accounting for most of the T3 in circulation (Norris and Carr, 2006). In the target tissue, T3 will be formed from T4 through the effects of diodinases. There are three major deiodinase enzymes in mammals (Figure 4), two isozymes, 5'-deiodinase type 1 (5'DI)(liver, thyroid and kidney)(most of the circulating T3 from the actions of 5' DII) and type II (5'DII)(central nervous system, placenta, skin, skeletal muscle and brown adipose tissue) known to convert T4 to T3, with some of the T3 entering the blood circulation. DII is considered more efficient that DOI and is the major role player in controlling intracellular conversion of T4 to T3 (Williams and Bassett, 2011). In a recent review Arrojo *et al.* (2013), confirmed the importance of DII-catalyzed T3 production and signaling and DII therefore critical in the T4-mediated feedback. A third deiodinase enzyme 5-deiodinase III (5D, DIII) removes iodine from the 5 (or 3) position on the inner phenolic ring and resulting in rT3 (diiodothyronine). The DIII type is known to suppress biological activity of thyroid hormones (mostly expressed in tissues with limited thermogenic responsiveness to T3). Therefore, both T4 (40%) and T3 can be inactivated by being converted to T2 or reverse T3 (rT3) by 5'deiodinase III. So, acting together, DII and DIII control the cellular T3 availability, largely independent of serum thyroid hormone concentrations (Bianco and Kim, 2006; Williams and Bassett, 2011). T3 and rTr3 can also be deiodinated to form 3,3' diiodothyronine (Figure 4)(Norris and Carr, 2006). In amphibians, two deiodinase subtypes are present, type II (DII), catalyzing the conversion of T4 to T3, and type III (DIII, known to inactivate T4 and T3 by conversion to T2 and rT3)(Huang *et al.,* 1999; Fort *et al.,* 2007).

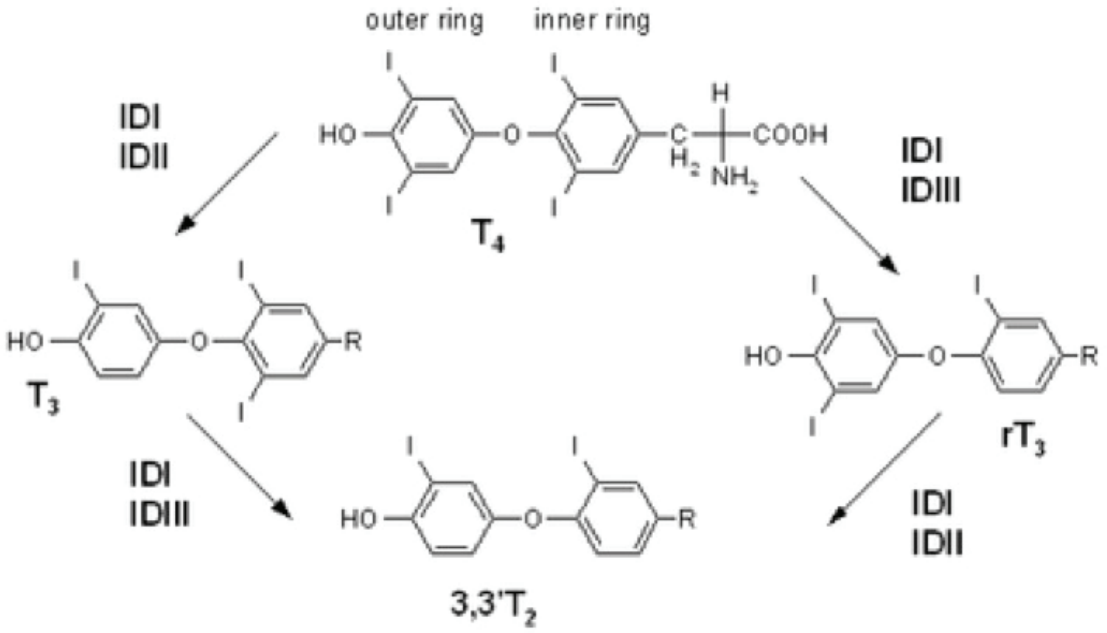

Figure 4. Conversion of T4 (thyroxine) by deiodinases (ID). Outer ring deiodination by type I and type II deiodinases produce T3 (biologically more active than T4). Inner ring deiodination by type I and type III deiodinases produce rT3 (biologically inactive). Deiodination of T3 and rT3 produce T2

2.2.4 Transport of thyroid hormones

The major thyroid hormone, T4 is transported in the blood bound to three different binding proteins. In humans 75% of T4 will bind to thyroxine-binding globulin (TGB), 15% will bind to transthyretin (TTR) and the remainder will bind to the plasma protein, albumin (Schussler, 2000; OECD, 2006). Thyroxine binding protein (TRG) is least abundant but with highest affinity for T4. However, TRG is only found in mammals and TTR in most vertebrates (Shi, 1999), with lower affinity to T4 but large carrying capacity. In amphibians, TTR is the major binding protein with a higher affinity for T3 than for T4 (Fort *et al.,* 2007). Most of the T4 in circulation will be bound whereas only 90% of the circulating T3 will be bound, making T3 more available to enter target cells (Norris and Carr, 2006; Williams and Bassett, 2011). Serum concentrations of T4 and/or T3 (total or free form) may be used as indicators of thyroid activity although interpretation may be complicated by inherent variation in plasma proteins and the fact that most of the plasma T3 may be derived from peripheral deiodination of T4 (mare a measure of DI activity that thyroid function).

It has been suggested that measurement of free T4 and T3 in relation to the transport protein, seeing that most of the TH will be bound is important (Fort *et al.,* 2007).

2.2.5 Metabolism of thyroid hormones

Similar to most other hormones, thyroid hormones (T3 & T4) are metabolized in the liver, either by deiodonation converting T4 to rT3 or by conjugation with sulfate or glucuronic acid and the excreted via the bile. Retention studies in frogs indicated that T4 is retained for longer periods than T3. Although T3 was found to be excreted in conjugated form, T4 was not excreted extensively in conjugated form (Fort *et al.,* 2007).

2.2.6 Thyroid hormone receptors and hormone action

Thyroid hormones are known to have gene expression effects in most cells in the body (Norris and Carr, 2006). Although thyroid hormones have a lipophylic nature it is generally predicted that TH will enter the target cell passively by diffusion (Friesema *et al.,* 2005) but with its hydrophobic core structures, free TH may enter the cell by carrier-mediated of facilitated transport (Oppenheimer, 1979; Williams and Bassett, 2011). Several membrane-bound transporter proteins are known to transport thyroid hormones across the cell membrane. As pointed out in an OECD report, chemicals that interfere with this facilitated transport may result in tissue specific disruption (OECD, 2006). Within the cytoplasm, TH bind to TH binding proteins (THBP), probably facilitating transport of TH to the nucleus (Shi, 1999). Thyroid hormones interact with nuclear receptors (TR that bind directly the thyroid hormone response element (TRE) in the nuclear DNA. Thyroid receptors are members of the superfamily of ligand-binding transcriptional factors (for example, steroids, retinoids and vitamin D)(Shi, 1999). It has been shown that two TR genes (diploid animals), alpha and beta, each encode unique receptors (Norris and Carr, 2006). However, *Xenopus laevis* known to be allotetraploid, possessing four TR genes (Fort *et al.,* 2007). TRα play a role during early stages of metamorphosis by suppressing T3 responsive genes, TRβ and basic transcription element binding protein (BTEB) (Brown *et al.,* 1995). Increased TRβ levels coincide with increases in TH levels and reach maximum concentrations during metamorphic climax. Of the two thyroid hormones, T3 binds to TRs with 5-10 fold higher affinities than T4. The possibility exist that T4 is first converted by 5'-deiodonase to T3, which then activate TRs (Shi, 1999). Inhibitors of 5'-deiodonases have been shown to inhibit the biological effects of T4, thereby implying that T3 is the active thyroid hormone (Becker *et al.,* 1997). Therefore, T4 acts as pro-hormone, which must be converted to T3 for the mediation of the TH actions (Bianco and Kim, 2006; Williams and Bassett, 2011). Important to note that DIII is expressed in fetal tissues where it acts as a barrier to prevent maternal TH access to developing young (Williams and Bassett, 2011). In mammals, birds and amphibians, the down regulation of DIII and subsequent upregulation of DII results in an increase in T3 production (Huang *et al.,* 2001).

TRs can bind to DNA as monomers, homodimers, and heterodimers formed with members of the thyroid-retinoid receptor subfamily (Shi, 1999). The best DNA-binding dimers are formed between TRs and RXRs (9-cis-retinoic acid receptors). These heterodimers are known to regulate the transcription of TH response genes and are recognized as the true mediators of the biological effects of TH (Shi, 1999). In the absence of TH, it is suggested that the heterodimer represses gene transcription through a co-repressor (Shi, 1999; Norris and Carr, 2006; Fort *et al.,* 2007).

TRα are expressed in most tissues of the body (OECD, 2006). TRα is also the most abundant TR in the fetal brain but since TRβ accounts for a small percentage of the total T3 binding in the brain, it received more attention because its expression coincides with a surge in fetal T3 levels (Norris and Carr, 2006). Both TRα and TRβ are expressed in the liver, the liver being recognized as a major site where T4 is converted to T3 (40%) by deiodination. Most of the T3 produced in the liver enters the blood circulation (Norris and Carr, 2006).

2.2.7 Thyroid hormone negative feedback on Pituitary gland

The negative correlation between serum thyroid hormone and serum TSH suggest that a negative control pathway at the pituitary and hypothalamus levels exist (Hollenberg, 2008). Specifically T4 correlated well (negative slope) to serum TSH levels. The fact that serum T3 did not correlate well with TSH levels suggest that T4 may be more important in controlling serum TSH than T3 (OECD, 2006). However, it was shown (using DII knockout mice) that DII is essential for regulation of the HPT axis and enables the pituitary to respond to changes in circulating T4 levels (Williams and Bassett, 2011; Arrojo *et al.,* 2013). It has been suggested that the receptor dependent negative feedback of TH on TSH, is facilitated by TRβ (OECD, 2006). Important to note that there are additional factors that may influence the TSH release from the pituitary. TRH from the hypothalamus increases TSH secretion, TH itself may control/regulate the gene encoding TSH (both alpha and beta subunits). Similarly, TH may regulate the expression of receptors in the pituitary. It is also known that TH (T3) may exert a non-genomic effect on TSH release. This phenomenon appears to be related to the ability of T3 to activate specific potassium channels in pituitary cells (OECD, 2006). Although earlier work place doubts on the feedback via the hypothalamus, recent work showed that the hypothalamus is sensitive to small changes in circulating TH (Abel *et al.,* 1999). The presence of a short-loop negative feedback, where TSH may control TRH is still uncertain. However, the potential for and ultra-short-loop where TSH play a role in its own release is supported by the fact that TSH receptors were found in human pituitary thyrotropes (Theodoropoulou *et al.,* 2000).

2.2.8 The role of Thyroid hormones in mammals

The thyroid hormones play key roles in regulating body development (brain, lungs, heart), energy metabolism and heat production but also in reproductive biology. Therefore, environmental contaminants that interfere with thyroid physiology or hormone action may disrupt the normal development and physiology adversely (OECD, 2006). In studies focussing on the role of the thyroid hormones in brain development (human and animals), it have been shown that normal thyroid function and hormone levels are required during certain key times during development (may differ between species) (Zoeller and Rovet, 2004; Norris and Carr, 2006; Zoeller, 2010).

2.2.9 The role of Thyroid hormones in non-mammalians (amphibians and fish)

It is now well-recognized that the role and importance of thyroid hormones in amphibian development is unparalleled in any other vertebrate group (Norris and Carr, 2006). However, although nearly 6275 anuran species (AmphibiaWeb, 2013) are known, most of the information regarding the details of metamorphosis come from a few species, *Xenopus laevis* (African clawed frog*), Rana catesbeiana* (bull frog) and *Rana pipiens* (northern Leopard frog) (Fort *et al.,* 2007). The dramatic and extensive changes associated with metamorphosis in amphibians are initiated and largely controlled by thyroid hormones. Thyroid hormones are required for most of the remodelling that will take place in transforming a basic aquatic organism into a terrestrial one. For example, the gastrointestinal tract is reorganized, limb development and tail resorption, restructuring of the cranial skeleton and physiological switches like the switch in hepatic nitrogen metabolism from ammonia to urea production all require thyroid hormonal action. Although mammals do not have such a complex life cycle, it has been recognized that metamorphosis is developmentally comparable to the mammalian post-embryonic organogenesis (Tata, 1994; 1998). It is well-recognized that the amphibians are separated phylogenetically from the other vertebrates (mammals, birds and fish) but that the conserved nature of the thyroid system (morphology and biochemistry) allows for the use of amphibians as general models to evaluate endocrine disruption with some extrapolation value to other vertebrates, including humans.

Temporally, anuran metamorphosis can be divided into three distinct periods, pre-metamorphosis, pro-metamorphosis and metamorphic climax (Shi, 1999; Fort *et al.,* 2007; Fort *et al.,* 2010). Pre-metamorphosis, refers to the period of embryogenesis and early tadpole growth and development basically in the absence of thyroid hormone (Figure 5), but with high expression of TRα and RXR (Figure 6). However, the initial development of the hindlimbs does occur during the pre-metamorphosis period (NF-stage 0 to 54). Pro-

metamorphosis, NF-stage 54 to 61, see extensive development of the hindlimb and differentiation of the toes. This period is characterized by the increase in TH and the up-regulation of TRβ expression (Figures 6). Metamorphic climax is the period, NF-stage 62 to 66, when endogenous TH is at its highest level and associated with rapid and extensive remodeling, including the resorption of the tail (Figures 5&6) (Shi, 1999).

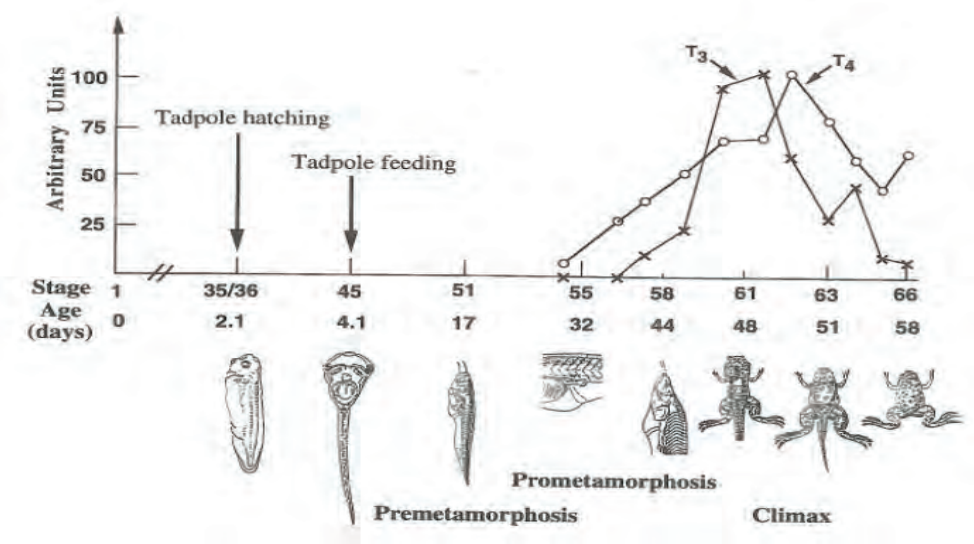

Figure 5. Developmental stage variation in plasma thyroid hormones (T4 & T3) concentrations in *Xenopus laevis* developing tadpoles during metamorphosis. Developmental stages follow the Nieuwkoop and Faber (1956) normal table. Reprinted with permission from Shi (1999).

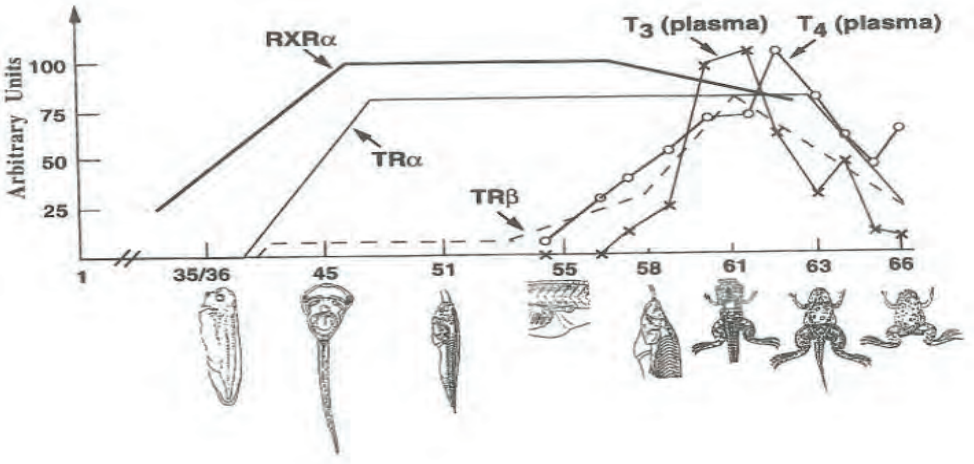

Figure 6. Developmental stage variation in the expression of thyroid hormone receptors (TRα and TRβ) and RXR (retinoid X receptor) in *Xenopus laevis* tadpoles during the developmental stages of metamorphosis. T4, thyroxine. T3, triiodothyrine. Reprinted with permission from Shi (1999).

A number of other hormones are known to play a role during metamorphosis, for example, prolactin (PRL), secreted early on during the premetamorphic phase are known to stimulate early development but inhibit metamorphosis (Hayes, 1997; Fort *et al.,* 2007) and PRL may be important in controlling high concentration of TH during metamorphic climax (Shi, 1999). Corticosteroids, on the other hand have been shown to accelerate TH induced metamorphosis (Denver, 1993; Hayes, 1995; 1997; Kloas *et al.,* 1997). A study by Hayes (1997) suggests that corticosteroid may slow down metamorphosis during the pre-metamorphic phase.

Clearly, the dependence of the amphibian metamorphosis on the HPT axis makes the development programme of tadpoles a potential target for environmental contaminants with the resultant modulation of rates of development. This potential screening approach was well recognized by environmental scientists (Kloas, 2002; Norris and Carr, 2006; Fort *et al.,* 2007; OECD, 2009; Pickford, 2010). A report, including 4 pesticides and 45 industrial chemicals as potential thyroid disrupting compounds in the environment (Brucker-Davis, 1998) highlighted the need for developing bioassays for TDC activity.

2.2.10 Evaluating Thyroid Disruption in Anurans

From the basic description of the anuran HPT axis and the functions of TH in anurans, it is clear that EDCs could potentially affect the HPT at several levels. Firstly, at the CNS (hypothalamus & pituitary) level, secondly, at the thyroid level and thirdly, TR level (OECD, 2006). In addition, TH transport and metabolism may be affected may be affected and add other hormonal systems that may affect thyroid activity, for example corticosteroids and prolactin. Physical environmental factors such as temperature and water level (density) may play a role in thyroid activity. So, clearly the complexity and extend of potential factors that may come into play, calls for a thyroid-bioassay designed to give a whole-organism response, especially at the first tier testing level. Thus it is clear, that the impact of EDCs on metamorphosis may occur at multiple levels. Amphibian metamorphosis therefore presents this opportunity to integrate morphological, biochemical and molecular effect on the thyroid system in a whole animal situation. The two primary developmental periods, pro-metamorphosis and metamorphic climax allows screening for modulation in metamorphic events. A battery of endpoints assessing disruption of normal development during metamorphosis has been used since the inception of the metamorphosis thyroid screen (OECD, 2006; Fort *et al.,* 2007; Kloas *et al.,* 2009; Pickford, 2010; Miyata and Ose, 2012). Initially the focus was on measuring the rate of tail resorption at the end of the metamorphic climax (Fort *et al.,* 2000). However, this approach did not consider the metamorphic events prior to the metamorphic climax and the natural variation in tail resorption could be high

because of the natural variation in endogenous TH. German and USA-EPA teams developed a 28 day *Xenopus* metamorphosis assay to include mainly the pro-metamorphosis phase of development but could include the early stages of the metamorphic climax phase. Multiple endpoints can be assessed and the OECD and USA EPA initiated a testing guideline, using *Xenopus laevis* (African clawed frog) as model to validated a metamorphosis, whole body bioassay, with inter-laboratory studies (Kloas *et al.,* 2002; Lutz *et al.,* 2005; Opitz *et al.,* 2005). The initial test period was shortened to 21 days and the histopathological analysis expanded (Grim *et al.,* 2009). The final XEMA guidelines were recently accepted for wider application (OECD, 2009). The selection of *Xenopus* laevis for a metamorphosis assay holds several advantages (Kloas, 2002; Kloas and Lutz, 2006). The fact that the full life cycle of *Xenopus laevis* happens in the water and that these frogs proved to survive a large water quality range make them good robust models to study EDCs in general. Larval stages are shown to be sensitive to changes in the aquatic environment and breeding of tadpoles from captive adults in laboratories all over the world underlines the value of *Xenopus laevis* as model species. One disadvantage is that the generation time is long, reaching maturity may take a year (Kloas and Lutz, 2006). An alternative amphibian species, the West-African clawed frog, *Silurana (Xenopus) tropicalis* has been suggested, since maturity may be reached in four months and metamorphosis could be used in a similar manner as proposed for *Xenopus laevis* (Kloas and Lutz, 2006; Mitsui *et al.,* 2006). However, it has been reported that Silurana tropicalis is not as robust as *Xenopus laevis* in the laboratory and during breeding programmes (Kloas and Lutz, 2006).

2.2.11 Short term gene expression

Besides the morphological and histological endpoints to evaluate thyroid disruption, more specific molecular endpoints relating directly toTH signaling in target tissues have been studied (Opitz *et al.,* 2006a). The main mechanism of TH action involves hormone binding to TH receptors (TRα and TRβ), resulting activation or repression (upregulation or downregulation)(increase or decrease in the mRNA levels of the gene) of gene transcription (Crump *et al.,* 2002a; Helbing *et al.,* 2003). The dramatic increase in endogenous TH at the end of the premetamorphic period result in an increase in the expression of the mRNA of the two thyroid TRs. Along with this upregulation in expression of the specific TRs, comes the activation of tissue-specific genetic programmes associated with the activity of TH (Helbing *et al.,* 2003). In this way, the expression of several genes in many tissues changes in response to T3 treatment. Although the natural expression of several TH responsive genes may be low during the later stages of the pre-metamorphic, the potential to respond to exogenous TH or TH-mimics makes the quantitative measures of mRNA of TRs valuable

biomarkers to examine the effects of TH modulation (Duarte-Guterman *et al.,* 2010; Duarte-Guterman and Trudeau, 2010; 2011). In mammals circulating hormone concentrations along with morphological and histopathological endpoints are used to assess the possibility of thyroid modulation, but the limited ability to obtain blood samples from tadpoles underlines the value of the analyses of TH-dependent gene expression as an alternative to assess thyroidal status (Opitz *et al.,* 2006a). For this approach exposure times ranging from 24 to 72 hours have been shown to result in adequate altered expression when compared to a control sample (Helbing *et al.,* 2003; Duarte-Guterman and Trudeau, 2010). The mRNA levels for as many as 79 genes were found to be altered in response to a T3 treatment in *Xenopus* tail tissue during natural metamorphosis (Veldhoen *et al.,* 2002; Helbing *et al.,* 2003). Exposing *Xenopus* tadpoles to the herbicide, acetochlor a total of 26 gene transcripts expressed I the tails (Crump *et al.,* 2002). Interestingly, acetochlor did not affect the expression of TRβ on its own significantly but did so when in mixture with T3 (Crump *et al.,* 2002). Therefore changes in expression levels of mRNA of TRβ directly or indirectly by modulating the expression profile of TH (T3) may be a valuable biomarker to screen for thyroid modulation effects, especially in combination with morphological and histopathological biomarkers. Goitrogen treatment of *Xenopus laevis* tadpoles from late pre-metamorphosis to early pro-metamorphosis resulted in increased TSH mRNA expression (Opitz *et al.,* 2009) but also in several TH responsive genes in the thyroid following a T4 treatment (Opitz and Kloas, 2010). Although histo-pathological examination and quantification of the thyroid gland seems to be the most sensitive measurement endpoint (Opitz *et al.,* 2006a), the quantitative gene expression approach could be used after a short exposure period in parallel, adding value to the understanding of the specific mechanism of action in play after a specific EDC exposure event.

Fish species have always been recognized as good bioindicators in aquatic toxicology and it is not surprising to also see the exponential growth in the scientific literature in EDC studies including fish species as model systems to assess and understand the interaction of environmental chemicals with the endocrine system (Kime, 1998). In terms of all the all the vertebrate species, fish represent close to half the species diversity and inhabit a wide range of environments in freshwater-, brackish- and marine aquatic systems (OECD, 2006). However, most research on the endocrine system and HPT axis in particular, in fish has been focused on teleost (bony) fish. Well-known in the EDC related literature, are the small fish model systems that have been developed as laboratory models to assess EDC activity in the aquatic samples or test/screen manmade chemicals for endocrine disrupting activity (Kime, 1998).

Similar to other vertebrates, including mammals, thyroid hormones hormones play an important role in a wide range of physiological functions and affect target cells in most tissues of the body. Apart from being involved in the maintenance and control physiology of a wide range of systems, the thyroid also plays a major role in the growth and development, including metamorphosis, in fish. The components of the thyroid axis have largely been conserved across vertebrates although functional differences may occur between species (Brown *et al.,* 2004). The thyroid tissue in most teleosts is scattered (non-encapsulated) in the pharyngeal (basi-branchial) region although isolate (ectopic) follicles may occur in other organs (Norris and Carr, 2006). As in all vertebrates thyroid tissue consists of follicles with a simple epithelium surrounding and external colloidal area containing proteinaceous colloid. The basic biosynthesis of THs and TG is similar to that reported for other vertebrates, including humans. However, unlike other vertebrates, iodide is obtained not only through the diet but also from water passing over the gills (Brown *et al.,* 2004; Norris and Carr, 2006; OECD, 2006). In fish (in contrast to mammals), a plasma protein (pre-albumin protein) actively binds iodide (Eales, 1990; Brown *et al.,* 2004) and plasma iodide levels are generally higher in fish than in other vertebrates. In fish, the control of the biological active T3 happens mainly in the peripheral tissue where T4 is transformed to T3 through deiodination. Although this is also the case in mammals, it seems that in fish T4 synthesis (serum T4) and tissue T3 are even less associated (OECD, 2006).

Similar to mammals, the thyroid is mostly controlled through the HPT axis, although the fish differ from other vertebrates in that the adenohypophysis of the pituitary is directly innervated from the hypothalamus and the portal system between the hypothalamus and pituitary does not exist (Eales, 1990; OECD, 2006). Functionally it is agreed that the mammalian HPT and fish HPT are similar in many respects but that that in the mammalian system the brain-pituitary-thyroid axis plays a stronger role in the control of both T4 and T3 levels, whereas in fish it is not the case and tissue conversion of T4 to T3 through deiodination plays an important controlling role. The HPT axis therefore largely ensures homeostasis of T4 in fish (OECD, 2006). The implication is that no single biomarker relates to the status of fish thyroid function (although may also apply to other vertebrates) and to assess xenobiotic effects on fish thyroid function it is recommended that a battery of tests are used to screen for disruption at several levels. Limited comparative information are available on the thyroid hormone receptors of fish, but two isoforms, TRα and TRβ, very similar to mammals and amphibians have been isolated form tissues (OECD, 2006). Expression studies mapping variation in temporal and tissue expression of these TRs in fish needs more study. This will allow for using changes in expression of TRs as result of xenobiotic exposures in short term bioassays more extensively.

2.2.12 Chemicals with thyroid disrupting activity

In reviewing the HPT axis, it becomes clear that the system is highly conserved in all vertebrate classes but indications are that the metabolism of xenobiotic chemicals and therefore exposure effects may differ considerably among vertebrates. Simple extrapolations from one vertebrate class to another are therefore cautioned against (Kidd *et al.,* 2013). Although it is true that most of the initial research focus was on reproductive related disruption caused by suspected EDCs, recently it has been recognized that disruption at various levels in the HPT axis may be linked to thyroid disorder in humans and wildlife. Case studies coming from mammals as well as non-mammalian vertebrates are growing exponentially (Kidd *et al.,* 2013). Evidence making a direct link between chemical exposure and thyroid disruption mostly comes from laboratory based studies, rather than wildlife studies or human epidemiological studies. Rodent models were used mostly to evaluate chemicals for thyroid disruption in mammals and humans (Kidd *et al.,* 2013). In both human and wildlife species studied, data obtained showed that long term exposure to TDCs may result in the lowering of free and total T3. According to the WHO (2013) report, a large portion of the non-mammalian research includes amphibian models, for example *Xenopus laevis*, due to the important role of thyroid hormone in controlling metamorphosis. These studies confirmed that environmental chemicals may act as thyroid disruptors and be linked to thyroid disorders in wildlife supporting the suspicion of such a link in disorders observed in humans.

Since the first suggestions that man-made and natural chemicals in the environment may disrupt the normal functioning of the endocrine system, many chemicals have been added to the list of concerned chemicals. Chemicals suspected to act as endocrine modulators (EDCs in general) belong to diverse classes linked to agriculture pesticides and fertilizers), waste water treatment (WWTP) effluents (including natural hormones, household products and pharmaceuticals), industrial effluents (Kidd *et al.,* 2013)(Table 1). These chemicals come from a variety of sources entering the environment. Some of these chemicals may be persistent in the environment and although may occur in low concentrations, may bio-accumulate through food webs to high concentrations in wildlife and humans. Moreover it has been suggested that different life cycle stages may be more sensitive, for example, developing fetus/embryo and juvenile stages may be particular sensitive windows for exposure with irreversible organizational effects (Guillette *et al.,* 1995; Hartoft-Nielsen *et al.,* 2011; Kidd *et al.,* 2013). Other EDCs may be less persistent or persistent enough to reach humans and wildlife but be less likely to bioaccumulate. Chemical behavior in the environment, either individually or in mixture with others complicate the exposure further. The WHO (2013) report group chemicals acting as EDCs into eleven

classes based on origin or chemical characteristics. More specifically, concern has increased regarding selected chemicals that specifically disrupt the thyroid axis, thyroid disrupting chemicals (TDCs). Lists and tables of examples of these chemicals have been published (Brucker-Davis, 1998; DeVito *et al.,* 1999; Boas *et al.,* 2006; Zoeller *et al.,* 2007a; Crofton, 2008; Boas *et al.,* 2009; Pickford, 2010; Hartoft-Nielsen *et al.,* 2011). Crofton (2008) has tabled suspected TDCs according to mechanism of action (see Table 1).

Although Brucker-Davis (1998) listed a comprehensive list of chemical with potential thyroid disruptive activity, many of these needed to be retested to confirm the thyroid disruption as defined under the broader definition as an EDC. Chemical classes that have recently been mentioned as TDCs include, the polychlorinated biphenyls, PCBs), Dioxins, Brominated flame retardants, Phenols, Phtalates, Pesticides, Parabens, Isoflavones, several metals and a few lesser-known chemicals (UV filters, perfluorinated chemicals, styrene, thiocyanate and nitrate). Although WWTP effluents have been mentioned as potential sources of TDC in several studies the influent source wastewater showed thyroid disruption but the treated effluent did show significant activity (Gracia *et al.,* 2008; Jugan *et al.,* 2009; Li *et al.,* 2010). Boas *et al.,* (2006), Crofton (2008), Hartoft-Nielsen *et al.,* (2011) and Jobling *et al.,* (2013) identified the potential points of action in the HPT axis according to mechanism of action (Figure 7).

In spite of the exponential increase in our knowledge regarding thyroid disruption activity of chemicals, there remain a substantial gap regarding the data to support linkage between chemical exposure and modulated thyroid function in wildlife and humans (Woodruff *et al.,* 2008; Jobling *et al.,* 2013).

Table 1. Classification of thyroid disruption classes, based on mechanism of action. Chemicals suggested to modulate or disrupt through a particular mechanism of action are include. Table redrawn from Crofton (2008). * References given in Crofton (2008)

Class	Mechanism	Response	Chemicals*
Iodine transport	Inhibit NIS	Decreased synthesis of T3 and T4	Perchlorate, Chlorate, Bromate
Synthesis Inhibitors	Inhibition of TPO	Decreased synthesis of T3 and T4	Methimazole, propylthiourea, amitrole, mancoceb, isoflavones, benzophenone 2, 1-methyl-3-propyl-imidazole 2-thione
Transport disruption	Altered binding to serum transport proteins	unknown	Hydroxyl-PCBs, EMD 49209, pentachlorophenol
Hepatic Catabolism	Upregulation of gluco	Increased bilary elimination of T3 and T4	Acetochlor, phenobarbital, 3-methylcholanthrene, PCBs, 1-methyl-3-propyl-imidazole-2-thione
Sulfo-transferases	Inhibition of sulfo-transferases	Decrease sulfation of THs	Hydroxlyted PCBs, triclosan, pentachlorophenol
Deiodinases	Inhibition of or upregulation of deiodinases	Decreased peripheral synthesis of T3	FD&C Red dye 3, propylthiouracil, tetrabromobisphenol A, actyl-methoxycinnamate,
TR effects	Direct or indirect alterations in TR-TRE binding	Altered activation of TH dependent gene transcription	Tetrabromobisphenol A, Bisphenol A, hydroxyPCBs

Apart for the direct effects in the HPT axis, several chemicals may indirectly influence thyroid functioning through effects on the uptake of iodine, for example perchlorate (Boas *et al.,* 2006).

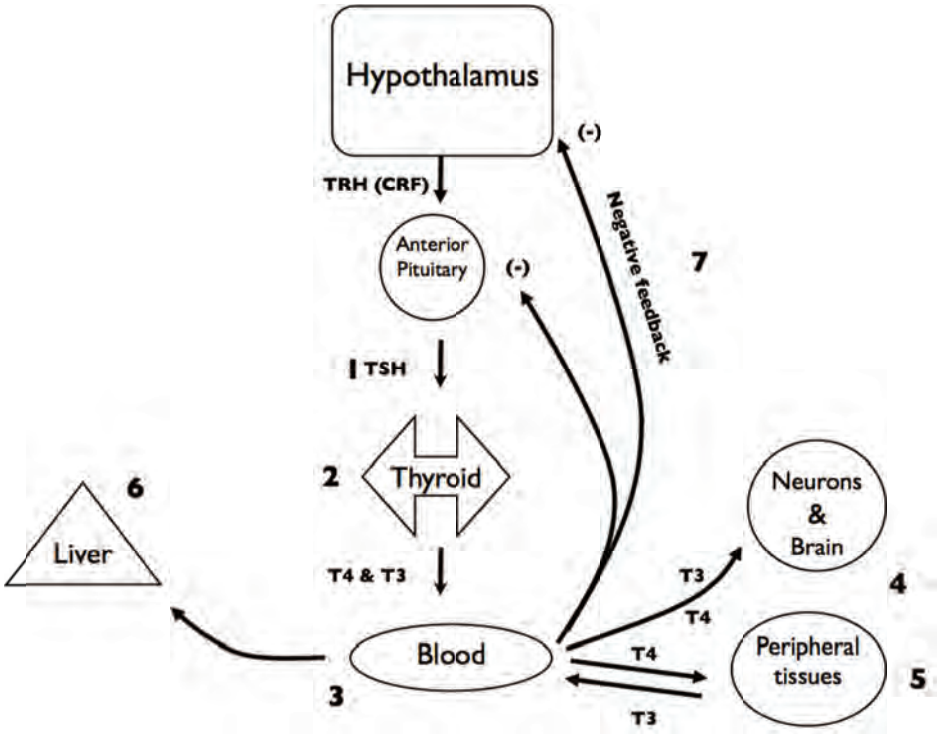

Figure 7. Possible sites of affect (mechanism of action) of environmental chemicals on the hypothalamus-pituitary-thyroid (HPT) axis. Interference with, 1) TSH secretion and receptor binding, 2) Synthesis of thyroid hormones: interference with NIS, TPO, deiodinases, 3) Transport proteins, 4) Cellular uptake mechanisms, 5) Peripheral deiodinases, TH receptors, 6) Metabolism of THs in the liver and excretion in the bile. TRH, thyrotropin releasing hormone. CRF, Corticotropin-releasing factor. The information in the figure is based diagrammes presented in several recent reviews (Boas *et al.,* 2006; Crofton, 2008; Jobling *et al.,* 2013)

From the initial testing and screening results it becomes evident that many chemicals may disrupt thyroid function but understanding this link may not be possible by evaluating only serum hormone levels (Jobling *et al.,* 2013). Therefore, it is recognized that the development and validation of extended series of bioassays to answer questions about the very specific mechanism-of-action involved in the disruption phenomenon is needed to strengthen the evidence that wildlife and human populations are threatened with thyroid disruption. Although there is evidence from laboratory based studies and limited information from wildlife studies (many wildlife species remain unstudied), the direct data supporting an environmental link to human thyroid disruption needs more study (Woodruff *et al.,* 2008). Considering the importance of thyroid hormones during development and adult physiology, the large knowledge gaps and potential population-wide impacts in wildlife and human populations is remains a matter of urgency to increase research capacity and address the knowledge gap (Jobling *et al.,* 2013).

In South Africa the situation is no different to the world-wide situation, but being a fast developing country in Africa additional concerns listed for the developed world may apply. Several chemicals or effluent types that end-up in our water resources have been suggested in the literature to have thyroid disrupting activity (Brucker-Davis, 1998; Crofton, 2008; Boas *et al.,* 2009; Hartoft-Nielsen *et al.,* 2011; Kidd *et al.,* 2013), for example, jet fuels and explosives (Brucker-Davis *et al.,* 2002; Tietge *et al.,* 2005a; Goleman and Carr, 2006; Carr *et al.,* 2008), several pesticides and herbicides (Brucker-Davis, 1998; Crump *et al.,* 2002a; Carr *et al.,* 2003; Sullivan and Spence, 2003; Hayes *et al.,* 2006) and environmental water (Fort *et al.,* 1999a; Bogi *et al.,* 2003; Jugan *et al.,* 2009).

3 CHAPTER 3: APPLYING THE *XENOPUS METAMORPHOSIS* ASSAY (XEMA)

3.1 Introduction

The *Xenopus* metamorphosis assay (XEMA) is designed as an *in vivo* screening tool to identify chemical substance in aquatic systems, which may interfere with the normal function of the thyroid endocrine system (OECD, 2009). Although this bioassay uses an amphibian system, the conserved structure and functions of the hypothalamic-pituitary-thyroid axis (HPT) makes it a generalized vertebrate model at the screening level (Tier 1). The dependence of amphibian metamorphosis on the functioning of the thyroid is a well-studied model (Shi, 1999) showing responses to substances active within the HPT axis during the developmental window of the organism. Within this framework, anti-thyroidal compounds expected to delay or inhibit the normal metamorphic developmental programme, whereas compounds that mimic or enhance the biological action of thyroid hormones will accelerate metamorphosis resulting in higher developmental stages than would be expected during the normal development.

Development, of *Xenopus laevis* tadpoles, based on morphological characters, has been divided into 66 stages by Nieuwkoop and Faber (1956) (NF-stages). NF-stage 1 represents the fertilization of the egg, followed by embryonic development during stages through to 35/36 when hatching takes place. Postembryonic development continues from NF-stage 37 through NF-stage 66 and feeding starts at NF-stage 45. The postembryonic development is divided into three distinct periods (based on morphological characters). The premetamorphic period (NF-stages 37-53) is characterized by rapid growth, minor morphological changes, low thyroid hormone levels (Shi, 1999). Thyroid formation starts around NF-stage 49 and functional gland activity around NF-stage 53/54. Accordingly, TH levels are low during premetamorphosis and it is only when THs start to increase that differentiation and development of TH responsive elements, for example the hindlimbs, occur. This marks the onset of the prometamorphic period (NF-stages 54-60). Touart (2002), in an initial USA-EPA DRP report pointed out that an assay falling in the premetamorphic period would be ineffective in detecting modulations of the thyroid pathways and that at the other end of the developmental programme, at metamorphic climax (NF-stages 60-66), TH-dependent morphogenesis requires very high TH levels and therefore expected to be a relative insensitive period (requires high levels inhibition or stimulation). Moreover, it has been suggested that the transition period from pre- to pro-metamorphosis would be particular sensitive to perturbation of the TH pathways because the exposures will be initiated with tadpoles that are initially naïve to TH. Effects of inhibition or stimulation are expected to be enhanced when initiated during the pre- /pro-metamorphic transition period (Touart, 2002). XEMA is therefore designed to start exposure at the onset of NF-stage 51

when endogenous THs are relatively low and continued into the pro-metamorphic phase when TH mediated development takes place. Since the pro-metamorphic period is expected to last approximately 20 days (Nieuwkoop and Faber, 1956) an exposure period of 21 days were agreed upon. The primary endpoints include, developmental stage, snout to vent length, body length, tail length, wet weight, hindlimb length, thyroid histology and daily observations of mortality (OECD, 2004) (see Table 2)

3.2 Methods

3.2.1 Xenopus Metamorphosis Assay (XEMA)

3.2.1.1 Test species

As an initial test species *Xenopus laevis* (Figure 8) has been chosen as the anuran species that meets the criteria of selection for a Tier I screening assay (Touart, 2002). *Xenopus laevis* is routinely cultured in laboratories in South Africa and around the world, and is easily obtainable from commercial suppliers (locally and internationally). Because this species occurs in the natural water of Southern Africa, collection of adults and maintenance in captivity (must be under a Nature Conservation permit) is also possible.

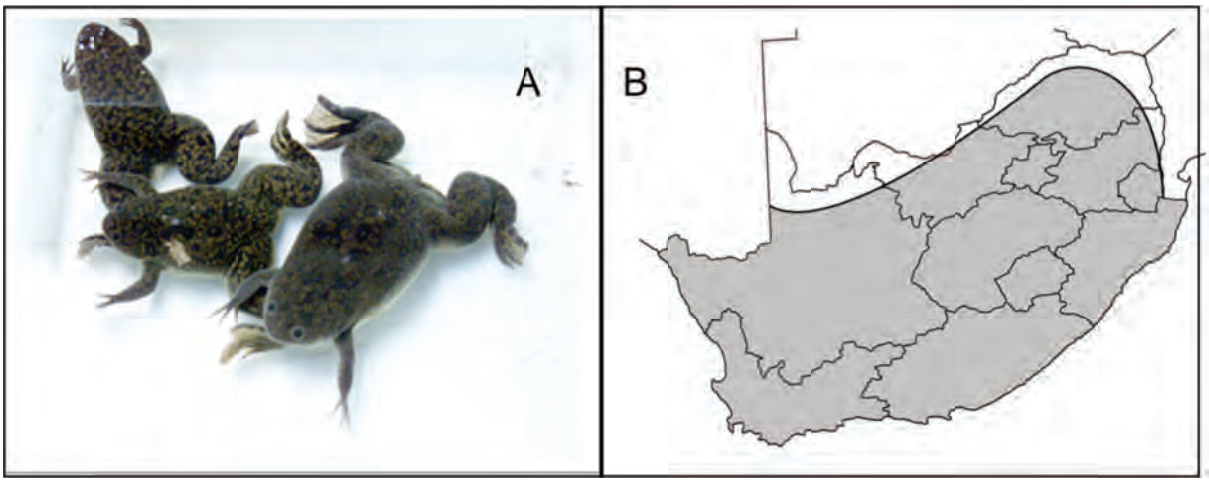

Figure 8. (A) African Clawed frog, *Xenopus laevis*. (B) Distribution of *X. laevis* in Southern Africa (After Du Preez & Carruthers, 2009).

In the laboratory, reproduction can be induced throughout the year (although not always the case in wild caught animals, Van Wyk (unpublished data) using human chorionic gonadotropin (hCG) injections. Larvae (tadpoles) were reared to the required NF developmental stage in large numbers (for details see below). Larvae used in XEMA should be from in-house breeding rather than collected from natural ponds or other sources.

3.2.1.2 Chemical Testability and Exposure systems; Semistatic vs. Flow-through systems

XEMA is based on an aqueous exposure system. The chemical compound is therefore introduced into the exposure tanks ether via a flow-through system or by adding a volume from a stock solution, in the case of the semi-static exposure system. When using a solvent, specific solvent control tanks are necessary. Since the physicochemical properties of compounds differ considerably, prior to testing, it is recommended that the OECD document on chemical properties (OECD, 2002) be consulted. If a successful test is not possible for the chemical using a flow-through test system, a static renewal may be used. According to the OECD (OECD, 2007c; 2009) a flow-through system is the preferred system if the system can accommodate the test chemical and maintain constant concentrations of the chemical.

Both static-renewal and flow-through diluter systems have been employed with XEMA (Kloas *et al.,* 2003; OECD, 2007c). In the initial testing of XEMA, the static-renewal system was used for a ring test, whereas the second ring test employed a flow-through diluter system. Since the static-renewal system is relative inexpensive and simple to setup compared to the flow-through system, the former approach is selected in laboratories without flow-through diluter facilities. Central in both systems are the use of glass, stainless steel and/or Teflon in water-contact components. Although suitable plastics could be used if it is known that these will not compromise the study. Exposure tanks should be glass aquaria ranging from 4 L-10 L in volume with a minimum depth of 10 to 15 cm. When setting up the system, replicates aquaria should be included (two to four replicates) (OECD, 2007d). In a flow-through system, it was suggested that the flow be 50 mL/min (3 L/hr, turnover of 2.7 hrs/tank) and in a static-renewal system, the exposure solutions should be renewed regularly (every second or third day). In the present study, we choose a much lower flow rate (17 mL/min, 1 L/hr, and turnover rate of 4 hrs/tank). *Xenopus laevis* tadpoles are known to be filter feeder in deep, but mostly static water and with a lower flow-rate when using environmental water the volume of water to transport become more practical (See Zandvliet study). The tanks should be randomly arranged a position to reduce possible positional effects, including variations in temperature and light intensity. Table 2 outlines the experimental conditions under which the XEMA (OECD, 2007b) should be used. Details of the specific set-up of the exposure system used in this study will be described below.

Table 2. Experimental conditions for the 21-day *Xenopus laevis* metamorphic assay (XEMA). Adapted from OECD reports (OECD, 2007a).

Test Animal	*Xenopus laevis* larvae	
Initial Larval stage	NF stage 51	
Exposure Period	21 days	
Larvae selection criteria	NF developmental stage and Total body length	
Test concentrations	Minimum 3 concentrations	
Exposure regime 1	Semi static with 3 water changes per week	
Exposure Regime 2	Flow-through, flow rate 1-3 L per hour *	
Dilution water/Laboratory control	Dechlorinated tap water (charcoal filtered) or RO water (add 2.5 g marine salt/10 L)	
Test vessel	4-5 L (10-15 cm standing height) glass aquaria (22.5 x 13 x16.5 cm)	
Laval density	20 larvae /test vessel (4-5 L)	
Replication	2-4 replicate tanks/ exposure group	
Feeding	Sera Micron (Sera GmbH, Heinsberg, Germany) ** 200 mg/d/tank (semistatic) (day 1-5); 300 mg/d/tank (day 6-21)	
Mortality in controls	< 10% overall	
Lightning	14 h light : 10 h dark; 600-2000 lux	
Water Temperature	21ºC ± 1ºC	
pH	6.5-8.8	
Dissolved Oxygen conc. (DO)	3.5 mg/L (>40% Air Saturation)	
Primary Endpoints	Mortality	Daily
	Developmental stage	Day 21
	Whole Body length	Day 21
	Tail length	Day 21
	Snout to vent length	Day 21
	Wet body weight	Day 21
	Hind limb	Day 21
	Front limb	Day 21
	Thyroid histology	Day 21

Thyroid Histology	Number	5 per replicate tank or 6 per group but NF stage matched
	Region	Head (lower jaw)
	Fixation	Davidson's fixative (48 hrs) then 4% buffered formalin

** OECD/EPA guideline state 3 L/hr but slower flow rate will also work since X. laevis tadpoles are deep-water filter feeders and swim in static water*

*** Sera Micron 30 mg/animal/day. Composition: 50.2% crude protein, 8.1% fat, 9.2% crude fiber, 5.0% moisture, and 11.9% crude ash. Ingredients: Spirulina algae, krill meal, shrimps,fish meal, nettle meal, wheat flour, paprika, alfalfa, brewer's yeast, Ca-caseinate, parsley, whole-egg powder, cod-liver oil, gammarus meal, sea algae meal, and mussel meal. Iodine content is 15 ± 1 ug/L (Coady et al., 2010). Alternatively see USA-EPA feeding (Degitz et al., 2005)*

3.2.2 Bioassay set-up used in the present report

For the current report we used (and evaluated) both semi-static and flow-through exposure systems. The latter approach was tested using a peristaltic pump (see Zandvliet study) and environmental water. All the other exposures conducted in this report were based on the semi-static XEMA exposure set-up.

3.2.2.1 Semi-static exposure test/assay

For the controlled experimental studies, we used 12 L glass tanks (with siliconed glass panels) as exposure tanks. Tanks were filled to a measured 10 L mark. Replicates (three) were included for exposure groups and tanks were placed randomly in a large stainless steel bath (Figure 9). We used lids to cover the tanks during exposure and air was bubbled into the tanks continuously (open ended tube without an air-stone) (Figure 9). Exposure tanks were set-up inside a climate control room (temperature control to ±1ºC).

The tanks and experimental water was replaced every 48 hours in the week (Monday, Wednesday & Friday) resulting a 96-hour exposure over the weekend (Friday to Monday). All chemicals were mixed into the exposure water (RO water with a marine salt added), an hour before the tadpoles were transferred to these fresh tanks and left to temperature acclimate. The same replacement tanks were used for each group and after each exposure session, the tanks were washed with soap and hot water, rinsed and washed with methanol and rinsed with RO water several times.

Figure 9. Series of duplicate glass tanks (10 L) with lids and air supply to aerate water. Experimental semi-static set-up was done in a climate control room (22ºC and 14 hr Light; 10 hr Dark). Glass tanks were placed inside stainless steel water baths.

3.2.2.2 Basic exposure conditions requirements for XEMA

Water temperature was maintained at 22ºC ± 1ºC as recommended by the OECD/EPA protocol (OECD, 2007d) (Table 2). The pH was maintained between 6.5 and 8 and the dissolved oxygen (DO) maintained at concentrations higher than 35 mg/L (>40% of the air saturation). These measurements were recorded on a daily basis.

The OECD/EPA recommendation suggest that good quality charcoal filtered spring water, know to facilitate normal tadpole development and growth could be used, provided a prior chemical analysis to ensure the absence of copper, chlorine and chloramines. Furthermore, the water should also be checked for background presence of perchlorate, fluoride, and chlorate (by-product of drinking water disinfectants) as all of these chemicals are known to affect the iodine uptake system and may therefore confound the outcomes of exposure. In the current study, we used RO water and added iodized marine salt (Cerebos®) (2.5 g/10 L), similar to the "Tropic Marine Meersalz" (2.5 g/10 L deionized distilled water) included in the initial SOP described by the German group (Kloas *et al.,*

2003). FETAX solution (Bantle, 1994; Bantle *et al.,* 1998) was used during the initial phase for breeding and hatching of eggs.

3.2.3 Adult care and tadpole breeding

Adult male and female *Xenopus laevis* frogs were housed in separate freshwater tanks. These conditions corresponded to ideal laboratory holding conditions described in the literature (Brown, 1970; Bantle *et al.,* 1998). Water in these tanks was replaced once a week after feeding (Trout pellets), with charcoal filtered tap water. Gutter down piping was placed in the tanks for frogs to hide in (enrichment). Water temperature in the room was maintained at 22℃ by air-conditioning and a light-dark cycle of 14:10 hours.

For in-house breeding of tadpoles three pairs were selected from the holding tanks and preconditioned by injecting hCG (Pregnyl®) (Donmed Pharmaceuticals (Pty) Ltd, South Africa) into the dorsal lymph sac (Brown, 1970; OECD, 2007e). Adult females initially received 150 I.U and males, 100 I.U. Sexes were house separately in 10 L aquaria and twelve hours later inspected to confirm the swelling of the cloaca labia in females and the presence of black keratin hooks on the forelimb (corresponding to the location of the epidermal breeding glands). A 10 L glass tank with lid was prepared for each pair by adding FETAX solution or tadpole culture water (RO water with salt added). An inner-mesh holder (to protect the eggs from the adults) was inserted and the water well aerated. The afternoon before the spawn/mating-night, the hCG-primed adult females received a second hCG injection (300 I.U.) to stimulate spawning and adult males received a second hCG (100 I.U.) to induce clasping (amplexus). The concentration of hCG used to induce spawning vary widely among researchers (Brown, 1970; Kloas *et al.,* 2003; OECD, 2004; Degitz *et al.,* 2005).

Together, a male and female pair was placed inside the mesh holder and into the prepared water-filled glass aquarium. The lid was secured and the tank place in a quiet (no disturbance), dark place for the night. Normally, eggs are laid during the night or early the next morning. The next morning, the tanks were carefully inspected for eggs or clasping. If mating was concluded and males released from clasping, the adults were carefully removed and placed in two separate holding tanks for recently bred adults. Eggs were inspected for fertilization and continuously aerated. Once developing eggs hatched and larvae surfaced, the developing larvae were removed from the mating tanks and place into a tank with well-aerated clean water (22℃, pH = 7 and light dark cycle of 14:10 hours). After spawning, eggs and developing tadpoles were reared in large (50 L) holding tanks containing 40 L tadpole culture water (RO water and marine salt) and a charcoal airlift filter. Feeding of

tadpoles started at days 4-5 PF (NF-stage 45). At this time when feeding started, small portions (just enough to lightly cover the surface of the tank) Sera Micron were given twice daily. A week after fertilization (day-8 PF) tadpoles were selected and place in smaller rearing glass aquaria (10-20 L) with an aerated biological filter system (containing activated charcoal and pebbles). Once moved to rearing tanks, tadpoles were continued to be fed twice a day (200-300 mg per day divided in two rations. Rearing medium was changed as soon as water became mucky. Tadpoles were reared under these conditions until they reached NF stage 48-50 (17-24 mm total length), and without any visible morphological deformities) (at ± 14-16 days PF). At NF-stage 48, tadpoles were selected and removed from the rearing tanks and place in exposure tanks (20 per tank). When the selected tadpoles reached NF-stage 51 (± 17 days PF) (Figure 10) a final selection was made.

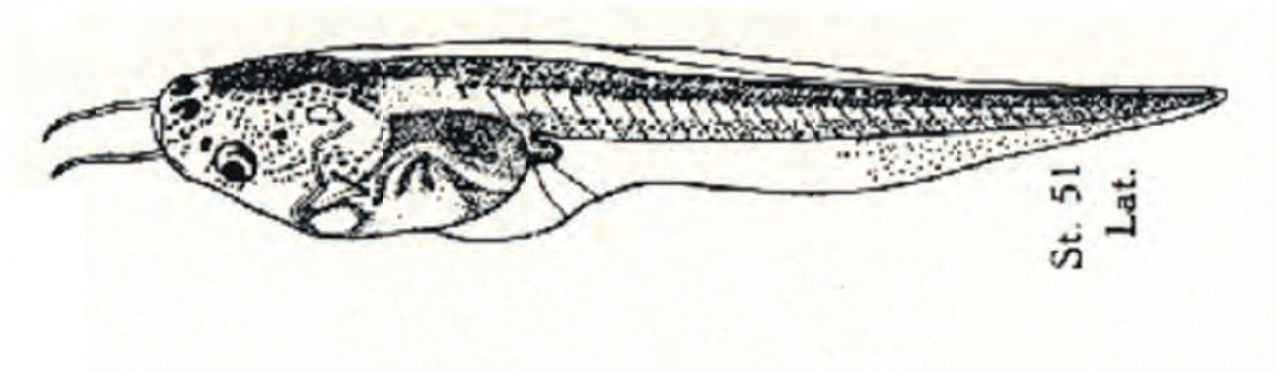

Figure 10. A *Xenopus laevis* tadpole at NF stage 51 (onset of XEMA) (after Nieuwkoop & Faber, 1956). Note the undifferentiated fore- and hindlimbs.

3.2.4 XEMA 21-day Exposure: general exposure regime

For the present validation study, we used the general experimental design of three replicates per chemical concentration (Figure 11). Initially we used 20 tadpoles per replicate and remove five/group at Day 7. However, subsequent to the initial exposure we only used the tadpoles that were exposed for 21 days (see Comment about 7-day tadpoles below). Therefore, the sample size increased to 60 tadpoles per exposure group (with three replicates). The exposure tanks were randomly assigned positions on the bench (Figures 9 & 11) in the climate-controlled room in order to account for possible variations in light intensity and temperature.

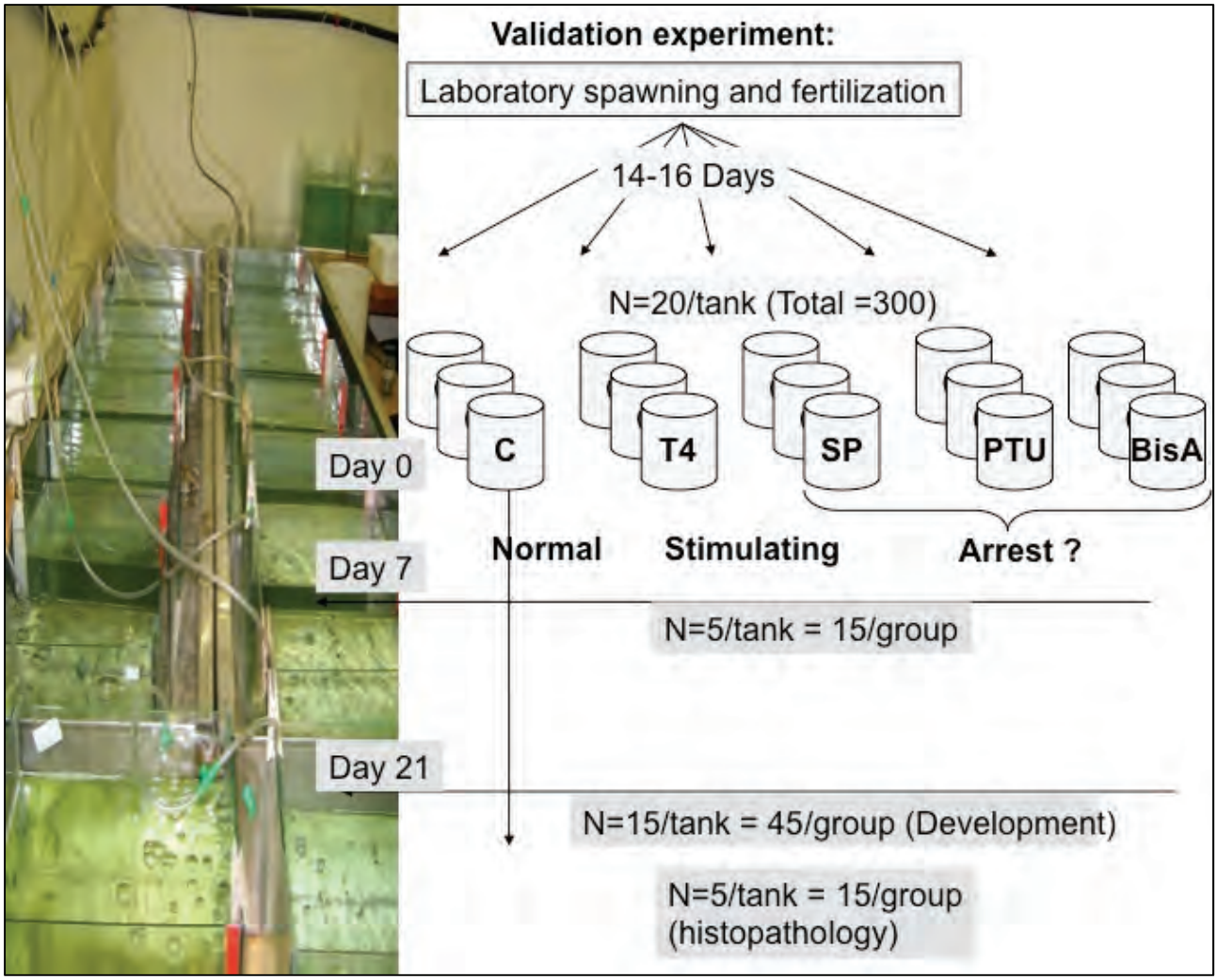

Figure 11. Summary diagram of the experimental procedures used in the laboratory validation study.

3.2.5 Test solutions for the Laboratory validation study

The initial validation study was set-up as a static renewal system. A double set of 10 L identical glass tanks were used. The day before the planned replacement clean tanks were filled with culture medium and allowed to temperature acclimate. The next day each tank was dosed with the selected exposure chemical and mixed well. Tadpoles were then carefully collected with a small-fish net and transferred to the tank containing the fresh test solution. [Note: It has been reported that latex gloves may be toxic effects on tadpoles (Gutleb *et al.,* 2001)].

Although the recommendation of the XEMA guideline is that at least three concentrations are included in a chemical study (with at least two, but preferably three replicates) we selected a single concentration for a known/control chemicals (and selected a concentration that gave good results) that were used in previous validation studies (OECD,

2004). In keeping with the recommendations, we used three replicates per exposure. The aim of this study was therefore to assess our capacity to conduct XEMA testing. In the case of Bisphenol A (BPA) it was decided to select a concentration from the literature suggested to have thyroid disruption effects and test it using XEMA comparing results to the controls (Iwamuro *et al.,* 2003) (Table 3).

Table 3. Exposure chemicals and concentrations used in the laboratory validation study

Chemical	Concentration	Action	Reference
Control Solvent Control	Buffered RO water DMSO < 0.01%	True endogenous negative control	
Thyroxine (T4)	2 µg/L	Stimulatory (positive) Control	(Kloas *et al.,* 2003)
Propylthiouracil (PTU)	75 mg/L	Inhibitory (negative) Control	(Kloas *et al.,* 2003)
Sodium perchlorate (sPER)	500 µg/L	Inhibitory (negative) Control	(OECD, 2007f)
Bisphenol A (BPA)	2.28 mg/L (10^{-5} M)	Hypothesized inhibitory effect	(Iwamuro *et al.,* 2003)

3.2.6 Exposure to environmental samples: case studies

3.2.6.1 Kuils-River – Eerste River catchment system

Water samples collected from the Kuils River – Eerste River catchment system were collected during October 2003 (late spring). Water samples were selected from five sites (Figure 12, Table 4). Three of the five sites (sites 2, 3 and 5) were situated downstream from wastewater treatment works (WWTWs), located at Bellville, Zandvliet and Macassar.

Fresh, water samples were collected weekly from the five selected sites. Water samples were collected and transported to the laboratory in stainless steel 25 L drums. Upon reaching the laboratory, water was split into two 10 L pre-cleaned glass tanks.

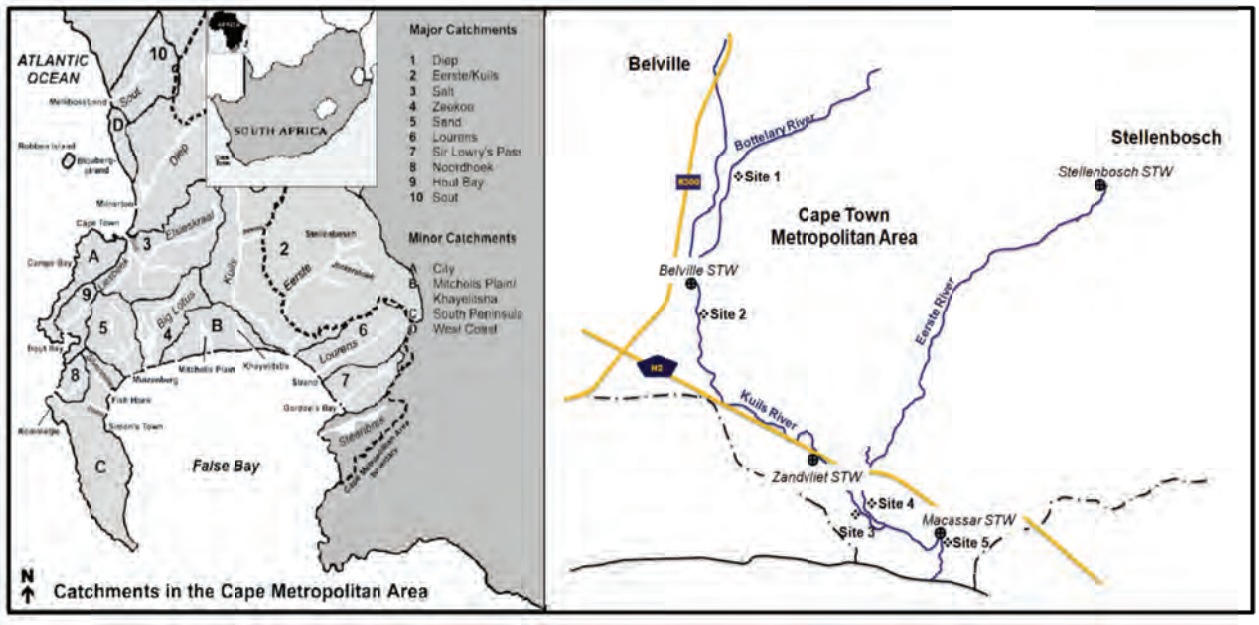

Figure 12. Sampling site in the Kuils River – Eerste River catchment system. Left panel displays larger Cape Metropolitan and Stellenbosch areas. Right panel indicates the water collection localities. Stars indicate the wastewater (sewage) treatment plants (STWs/WWTPs) discharging into the system.

Table 4: Description of sample points along the Kuils River – Eerste River catchment system

Sampling point:	Site name:	State of the Rivers status*	Physical description:
1	Bottelary River Amandel Road	Fish = good SASS = poor WQ = poor	Surrounding urban area Winery along upper reaches Storm water discharge pipes
2	Kuils River: Downstream of Bellville WWTP	Fish = poor SASS = unacceptable WQ = unacceptable	Downstream of WWTP discharge High levels of pollution (plastic/food/clothes) Housing – shacks to brick houses
3	Kuils River: Zandvliet WWTP	No data	Downstream of WWTP discharge
4	Eerste River N2 Freeway	Fish = poor SASS = good WQ = fair	Stellenbosch catchment Agriculture Stellenbosch WWTP discharge
5	Kuils-Eerste River Macassar WWTP Estuary	Fish = poor SASS = poor WQ = unacceptable	Downstream of WWTP discharge Estuary – outlet into ocean Situated close to beach

**River Health Programme Report (2005). State-of-Rivers Report: Greater Cape Town's Rivers. Department of Water Affairs and Forestry, Pretoria.*

Premetamorphic tadpoles (NF-stage 51) were randomly selected from a rearing holder tank and placed into exposure tanks (10 L) and exposed to undiluted water samples collected at selected sites along the Kuils River – Eerste River catchment system for 21 days. The twelve, 10 L tanks (replicate samples of a laboratory control and five river samples) were setup in a climate-controlled room, temperature 22 ± 1ºC and a light-dark cycle of 14 L:10 D hours. Tanks were cleaned and water exchanged every week (every seven days). The exposures were ended on day 21. The standard XEMA set-up was followed for maintaining the tadpoles and to process the exposed tadpoles after terminating the experiment (see above).

3.2.6.2 City of Windhoek Waste Water Treatment/Reclamation Plant

Water samples to assess the potential thyroid disrupting activity occurring within the treatment pathway of the City of Windhoek WWTP reclamation plant were collected during November 2008. Water samples were collected from four sites, distributed throughout the reclamation plan for the XEMA screening (Table 5).

Table 5. Water samples collected at the City of Windhoek (Namibia), Goreangab reclamation plant during summer, November 2008.

Sample Site	Site name	Key points	Description
Site 1	Final (GAC)	Effluents, Drinking water from NGRP	The final water purified with GAC before blended with treated surface water
Site 2	Raw Mix (RM)	Final Effluents Gammas STP, merged with Raw Goreangab Dam	The entry point located as the bottom of the plant, mixture with Goreangab and Gammas point
Site 3	Von Bach (VB)	Located near Okahandja	Surface water
Site 4	Black Rose (BR)	Highly populated Dense Areas	In the distribution system, this point is located within the Okuryangava area, with a high pollution and high density of people per square meter

Bi-weekly samples were collected in polyethylene 25 L drums at the Windhoek, Namibia location and send over-night to Stellenbosch University (Ecophysiology Laboratory) (1500 km) for exposure studies. A semi-static exposure approach was followed and water was

changed three times a week (Monday, Wednesday and Friday). Water was stored in a cool place on arrival, usually the day before the intended water change. Exposure tanks were replicated with 20 tadpoles NF-stage 51 tadpoles per 10 L glass tank (See Kuils River – Eerste River set-up). Exposures were ended on Day 21. The XEMA set-up as described previously was used to process the tadpoles.

3.2.6.3 Zandvliet, Cape Town, WWTP (Flow-through system)

Water (final effluent) was collected on a daily basis from the Zandvliet WWTP (City of Cape Town) (see Figure 12). Water was collected in clean 25 L plastic drums and transported to the laboratory (30 km). Water was collected on a daily basis. For the control groups buffered RO water (RO water supplemented with iodated marine salt, 2.5 g\10 L) was used. For the 50% effluent group, the collected water was split into two drums and diluted to 25 L by adding buffered RO water. Drums were left in the temperature-controlled laboratory to reach the environmental temperature of the exposure water (22ºC). For this study, a simple flow-through set-up (Figure 13) was used.

Figure 13. Simple flow-through set-up. An 18 channel peristaltic pump was used to control the flow rate. Replicate exposures were included for a Water Control (Buffered RO water), 50% diluted effluent and 100% (undiluted) effluent.

For the flow-through tanks we constructed glass tanks (22 x 14 x 16.5 cm) (Table 2) according to the OECD guidelines (OECD, 2007c;d). The glass-panels were silicone glued and a standpipe was mounted (through a Teflon stopper) centrally in the bottom-panel. This glass-standpipe was a 10 mm glass tube and adjustable to control the height (8-15 cm height) of the water chamber to 4 L. Tanks were positioned on a wooden shelf with holes drilled, to facilitate the external aspects of the individual down pipes to discharge into a ventrally mounted gutter taking effluent water to an effluent holder or into the laboratory wastewater drain (only environmental samples).

To create a flow-through, we used an 18-channel peristaltic pump (Figure 13). The suction tubes (two, replicates) were inserted directly into the respective 25 L holding drum. Although the recommended XEMA flow rate is 50 ml/min (3 L/hr; 2.7 hrs for a tank turnover) (Table 2) we used a slower flow rate (17 ml/min; 1 L/hour; 4 hrs for tank turnover when using replicates) to correspond to the static, deep-water conditions that *X. laevis* tadpoles will experience in the wild. Also, it allows for replacing the exposure drum with fresh water every 24 hours. Notwithstanding the low flow-rate, very little debris build-up was noticed in the tanks and the tanks could be cleaned using a syphon.

3.2.7 Exposure termination, tadpole measurements, and histological analyses

3.2.7.1 Day 7 removals (only in validation study)

During the standard XEMA exposure phase of 21 days, a subset of five tadpoles per treatment tank was removed for staging and body size measurement. Tadpoles were anesthetized in 2000 mg/L buffered MS-222 (tricaine methanesulfonate). Tadpoles were rinsed in water, blotted dry and weighed on an electronic balance to the nearest milligram. Total length (TL) and Snout to Vent Length (SVL) was measured using a ruler. Tadpoles were fixed in Davidson's fixative (Table 2) for 48 hours and subsequently transferred to buffered 4% formalin as preservative. The NF developmental stage was determined using the staging criteria described in Nieuwkoop and Faber (1956) and a dissecting microscope.

3.2.7.2 Day 21 removals (Test termination)

On Day 21 of XEMA, the remaining 15 tadpoles per group were removed from each exposure tank and anesthetized in 2000 mg/L MS-222. Tadpoles were rinsed in water, blotted dry and weighed on an electronic balance to the nearest milligram (Figure 14). Total length (TL) and Snout to Vent length (SVL) was measured using a ruler. Tadpoles were fixed in Davidson's fixative (Table 2) (OECD, 2007d) for 48 hours and subsequently transferred to buffered formalin as preservative. The NF developmental stage was determined using the staging criteria described in Nieuwkoop and Faber (1956) and a

binocular dissecting microscope. Measurement of hindlimb length (HLL) and forelimb length (FLL) were made using stereomicroscope (Leica) and image analysis software (Sigmascan, Systat Software Inc.) (Figures 14 & 15).

Five tadpoles (similar NF-stage) were selected from each treatment group (including replicates) (total 10 tadpoles) for histopathological analysis of the thyroid gland. [Note: the OECD guideline suggests the random selection of tadpoles for histological studies, but since this could result in the inclusion of tadpoles of different developmental stages quantitative measurements may not be valuable].

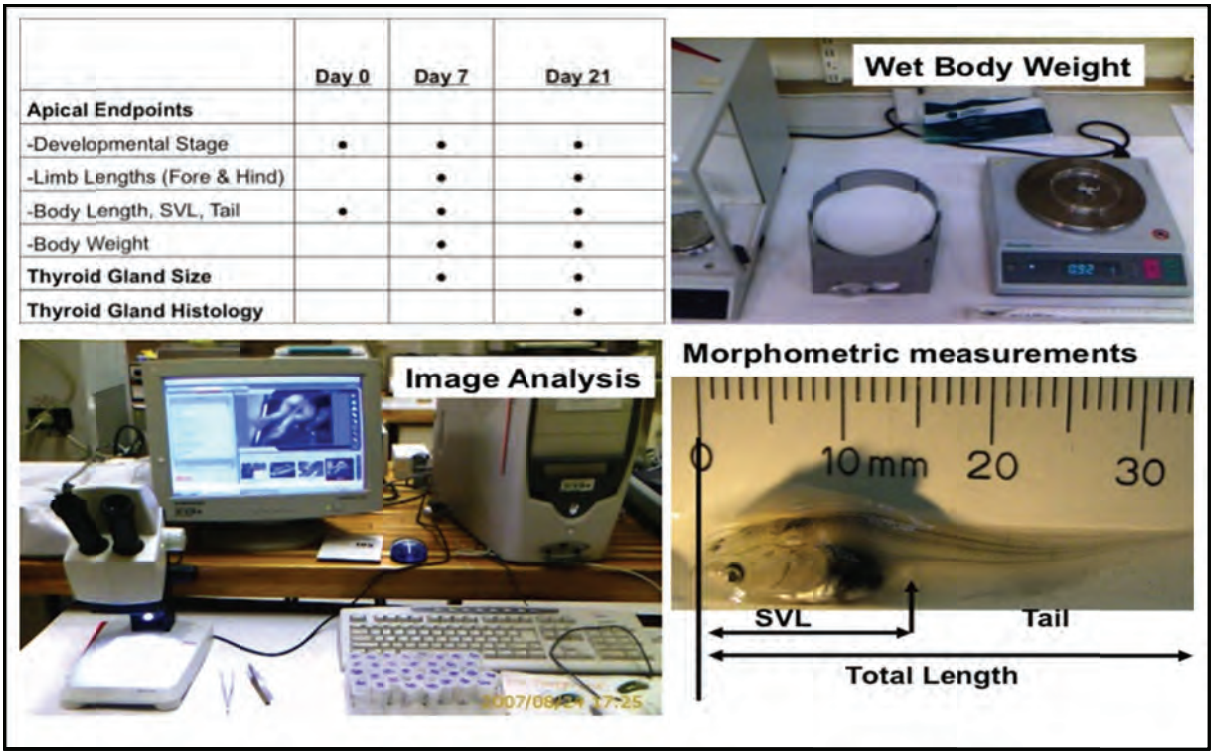

Figure 14. Summary diagram to show aspects of the post-exposure procedure. Time table with endpoints, electronic balances, ruler and binocular dissection microscope with camera and image analysis software to measure limb lengths and do NF staging.

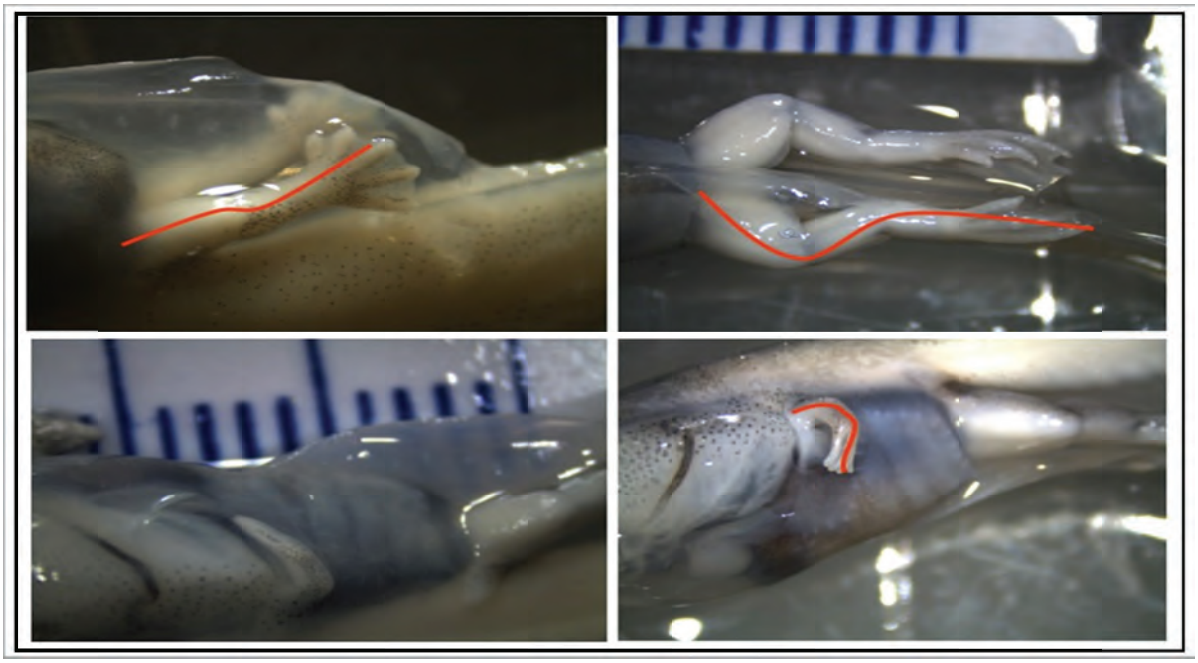

Figure 15. Measurements of limb with dissecting microscope. (Top): Hindlimbs, left NF-stage 55 and right NF-stage 60. (Bottom): Left, forelimb in atrium (NF-stage 57) and right, forelimb free (NF-stage 58).

The heads, containing the lower jaw was dissected from the tadpole body (head was decapitated, with a sharp blade, from fixed tadpole behind the eye (transverse section on caudal side) at the level of the heart) and subjected to routine histological procedures. Although the OECD guidelines recommend the use of an alternative procedure (using isopropanol and methyl benzoate as replacements 100% ethanol and toluene) (OECD, 2007c,e) we decided to revert to routine techniques. Since this procedure turned out to be time consuming, in subsequent studies, routine histological procedures were followed (Bancroft and Stevens, 1977). The tissue samples were infiltrated with paraffin wax (Histosec, 56ºC) and imbedded in the frontal plane in a manner that will facilitate the caudal surface of the tissue first. Alternatively, tadpole head samples were imbedded upright in such a manner that transverse sections were taken from the caudal to rostral side. Sections were inspected unstained and before wax removal, to evaluate whether the thyroid has been sectioned. Sections were mostly taken at 7-10 µm. Serial sections were taken once the thyroids have been located. Sections were mounted on albumin coated glass slides and dried in a 40ºC -drying oven. The dried slides were stained routinely using Hematoxylin and Eosin staining. Sections were dehydrated in a graded series of alcohol and eventually cleared in xylene and a coverslip mounted with a resin-based mounting medium (DPX) (Bancroft and Stevens, 1977). Histological endpoints recorded, included quantitative

variables (morphometric measurements), descriptive and qualitative (histopathology compared to an experimental control) included:

Quantitative variables:

- Epithelial cell height.
- Follicular cross-sectional area
- Thyroid cross-sectional area

Qualitative, descriptive variables:

- Follicular cell shape (cuboidal, columnar, tall)
- Follicular cell height (increased or decreased)
- Structure of the epithelium (single layer or stratified)
- Presence of follicular hyperplasia)
- Qualitative, severity variables
- Reduced or increased size of the thyroid gland
- Reduced or increased follicle size
- Follicular shape (regular, irregular, uniform)
- Colloid content (increase or reduction in colloidal area)
- Colloidal density (homogeneous or heterogeneous

Photographs (Figure 16) of the right thyroid glands were taken using a high power bright field microscope equipped with a digital camera. The digital images were used to measure epithelial height measurements. For each follicle (at least three selected for measurements), the heights of 10 epithelial cells were measured (taken from the base to the apical edge of the cell). The mean value for epithelial cell height was calculated for each individual (n = 5-10). Cross-sectional areas of the largest follicles were calculated. Similarly, using image analysis software, we calculated thyroid gland cross-sectional area. The OECD guideline suggests that that a severity grading is included (OECD, 2007a).

3.2.8 Statistical analyses

Median rather than mean values were used for developmental stage data. Normality and homogeneity of variance of the data was assessed using the respective Shapiro-Wilks and Levene's tests. Non-parametric data were analysed using Kruskall-Wallis ANOVA followed by Multiple Comparisons of Mean Ranks to assess pairwise differences whereas for parametric data, one-way ANOVA, followed by Tukey HSD Multiple Comparisons were performed when needed. The Mann-Whitney U-test was applied for single pairwise

comparisons of non-parametric data. Probability values of ≤ 0.05 were deemed significant. All analyses were performed using Statistica 10 (Statsoft Inc., USA).

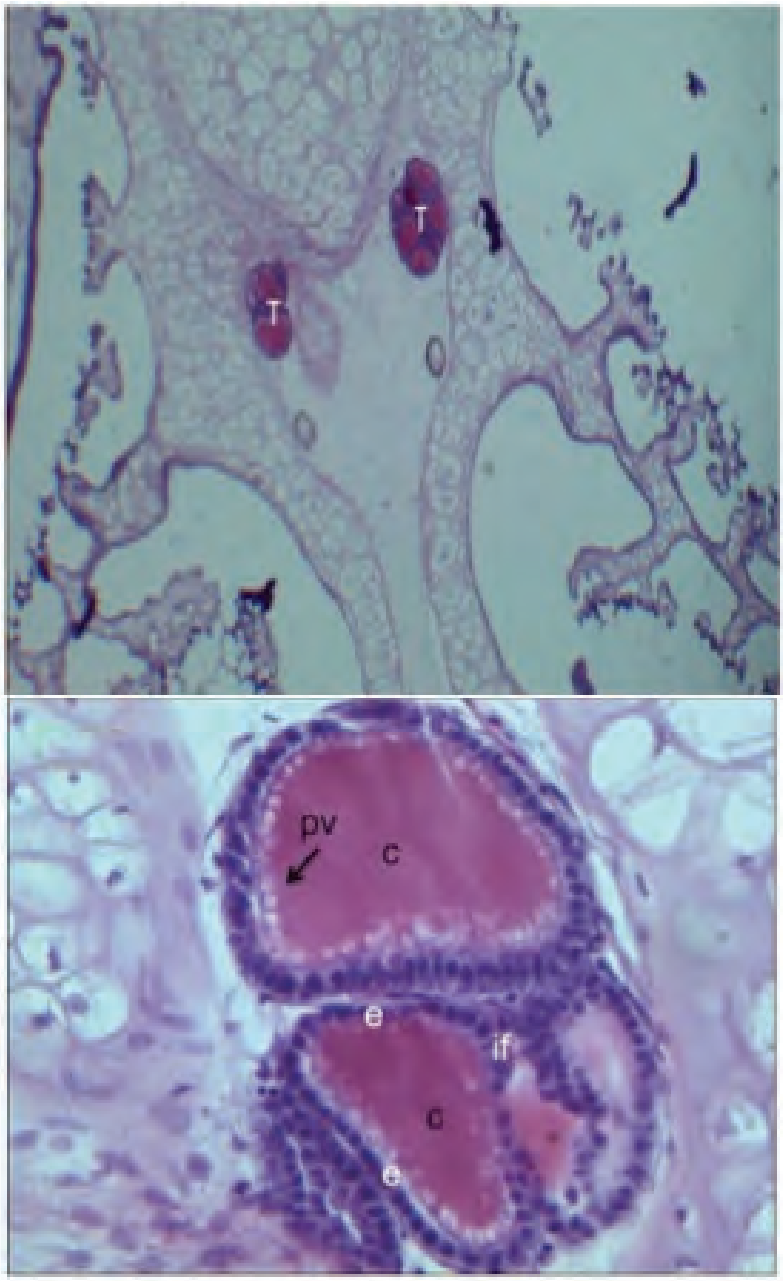

Figure 16. Histological section of the Xenopus *laevis* thyroid (paired structures). (Top). A low magnification (40x) light micrograph of a histological section through the hyoid region of a NF stage 57 tadpole, stained with Hematoxylin and Eosin. (Bottom). A high magnification (400x) light micrograph of a histological section displaying thyroid follicles in more detail. T = thyroid; c = colloid (pink); e=epithelium (purple); if=inter-follicular stroma; pv=peripheral vacuoles.

3.3 Results

3.3.1 Laboratory validation of XEMA

3.3.1.1 Tank effects and Mortality

All exposures were conducted in duplicate and tanks were randomly distributed in the exposure room. Since no tank effects were recorded, samples from duplicate tanks were subsequently pooled. No mortalities were recorded during the 7-day or 21-day exposures and exposure conditions were successfully maintained within the water quality guidelines expected by the XEMA set-up.

3.3.1.2 Variation in NF Developmental stages

Developmental NF-stage is one of the key biomarkers for the assessment of thyroid status in the XEMA set-up. The original XEMA set-up included several time frames, including 7-day and 14-day exposure periods. In this study, we initially compared the 7-day exposure to the 21-day exposure regime. From Figure 17 it is clear that within the first 7-day exposure period, developmental progress was slow and no significant (P>0.05) variation in NF-stage was found among the exposure groups. The exposures were initiated at NF-stage 51 and after seven days most of the tadpoles progressed to NF-stage 53 (still within the recommended target NF stage range, 53-55, OECD, 2007).

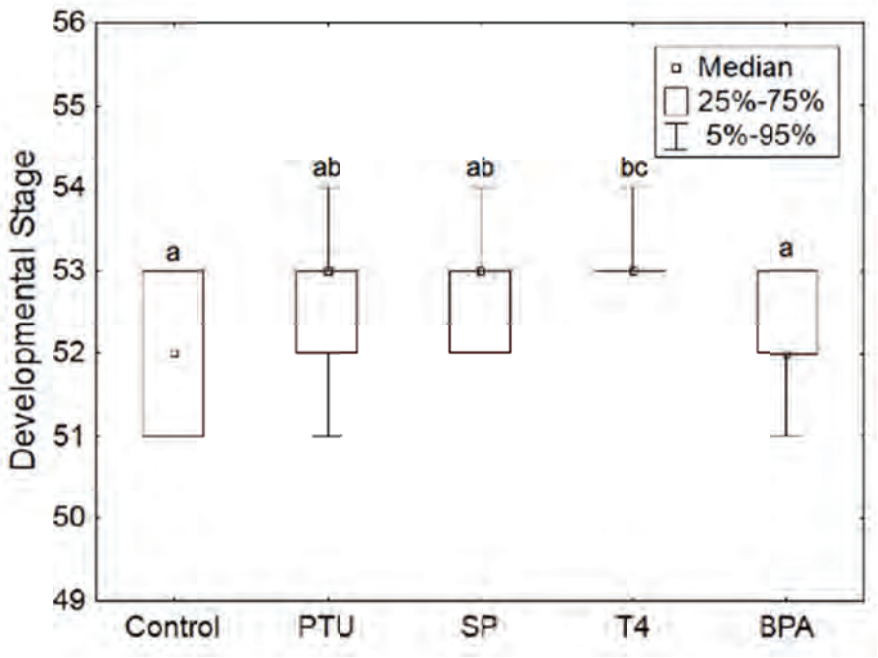

Figure 17. NF developmental stages observed in *Xenopus laevis* tadpoles exposed for seven days to buffered RO water, propylthiouracil, sodium perchlorate, thyroxine and Bisphenol A respectively. Dissimilar letters indicate statistically significant differences (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks).

This was not the case after the 21-day exposure (Figures 18 & 19). From Figure 18, it is clear that considerable variation occurred in development when taken over the 21-day exposure. The median NF-stage for the three control groups combined was NF-stage 56 (±2). Clearly the stage distribution was reasonably tight in most of the exposure groups (Figure 18). For this particular experiment NF-stage distributions of control chemicals (T4, PTU and PER) and the environmental pollutant, Bisphenol A, were compared to the exposure control.

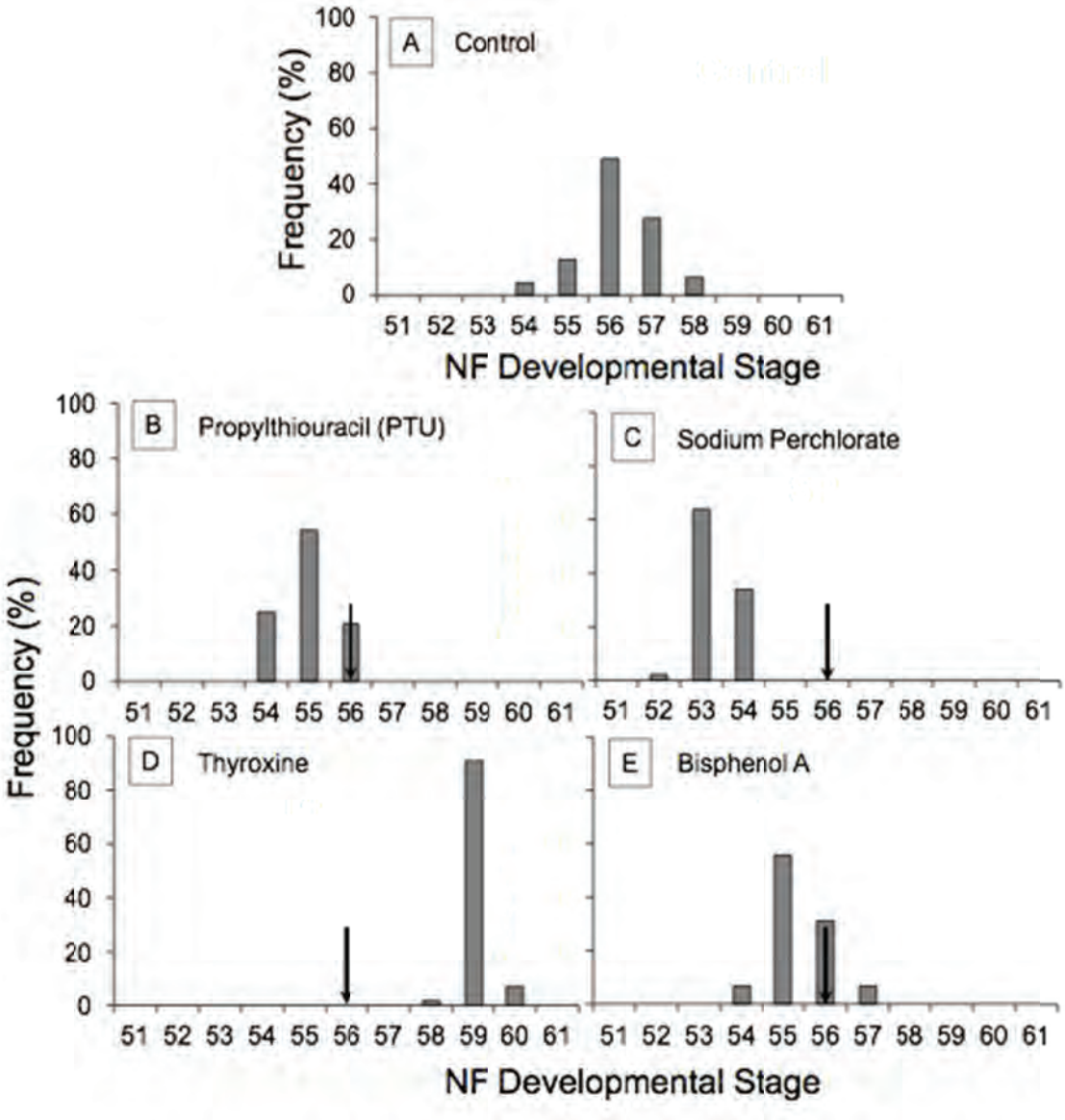

Figure 18: The frequency distributions of developmental stages (Nieuwkoop and Faber 1958) attained by *Xenopus laevis* tadpoles exposed for <u>21 days</u> to (A) buffered RO H_2O (i.e. Control), (B) propylthiouracil (PTU), (C) sodium perchlorate (PER), (D) thyroxine (T4) and (E) Bisphenol A (BPA). Vertical arrows indicate the median stage (NF stage 56) attained in the control exposure group.

Both PTU and sodium perchlorate (PER) inhibited the development of the tadpoles (Figures 18B and 18C respectively) when compared to the experimental control (Figure 18A). Figure 19 suggests that PER was a more potent inhibitor of tadpole development than PTU in this study. Thyroxine (T4), on the other hand, clearly enhanced development progressing towards the metamorphosis climax (Figures 18D & 19). Although the distribution of NF-stages in the Bisphenol A group was also within the negative and positive outliers, the shift (relative to the control) may suggest slight inhibition (but not significant, Figures 18E & 19).

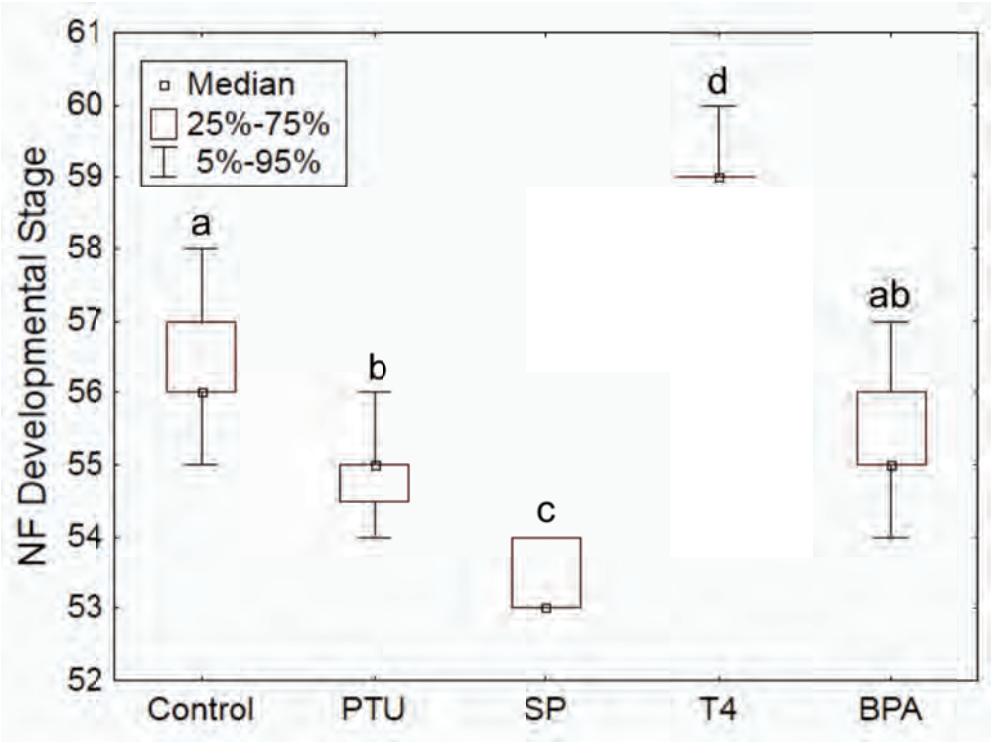

Figure 19. NF developmental stages observed in *Xenopus laevis* tadpoles exposed for 21 days to buffered RO water, propylthiouracil, sodium perchlorate, thyroxine and Bisphenol A respectively. Dissimilar letters indicate statistically significant differences (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks).

Considering the medians and confidence intervals for each group in the 21-day exposure (Figure 19) the inhibition (reported in most validation studies) caused by PTU (and PER) was confirmed, both being significant different ($P<0.05$) (Figure 19) from tadpole development in the laboratory control group. The NF-stage median for the thyroxin (T4) group was around 59-60 and well advanced towards completion if metamorphosis (NF-stage 66) (Figure 19). Tadpoles in the BPA group did not show a significant advance or delay in development when compared to the experimental control. Bisphenol A in this exposure showed a trend towards a similar delay, as was the case with the PTU exposed tadpoles (median NF-stage 55), but did not differ significantly from the control (median NF-56).

3.3.1.3 Variation in Hind- and Forelimb development

Forelimbs were not measured in NF-stage 52 tadpoles after seven days of exposure (too small). Hindlimb length did vary significantly among exposure groups after seven days (Figure 20). Hindlimbs were significantly longer in PTU, SP and T4 exposure groups when compared to the control group. The hindlimbs of the BPA group did not differ significantly from the control and PTU groups but did so when compared to the SP and T4 groups.

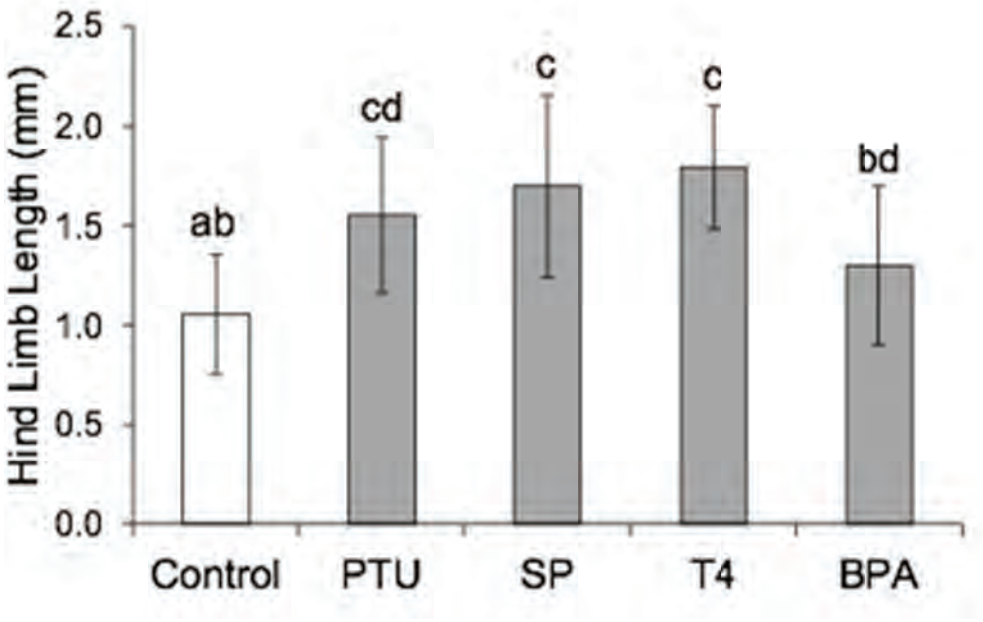

Figure 20: Mean ± standard deviation of the hindlimb lengths of *Xenopus laevis* tadpoles exposed for seven days to buffered RO water (Control), propylthiouracil (PTU), sodium perchlorate (SP), thyroxine (T4) and bisphenol A (BPA) respectively. Dissimilar letters indicate statistically significant differences (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks).

Both forelimb and hindlimb did vary significantly after 21 days of exposure. Forelimbs were significantly reduced in the perchlorate-exposed tadpoles (Figure 21). The tadpoles in the T4 group showed significant ($P<0.05$) longer forelimb length than the tadpoles from the control group. A similar trend was observed in the hindlimb (Figure 21).

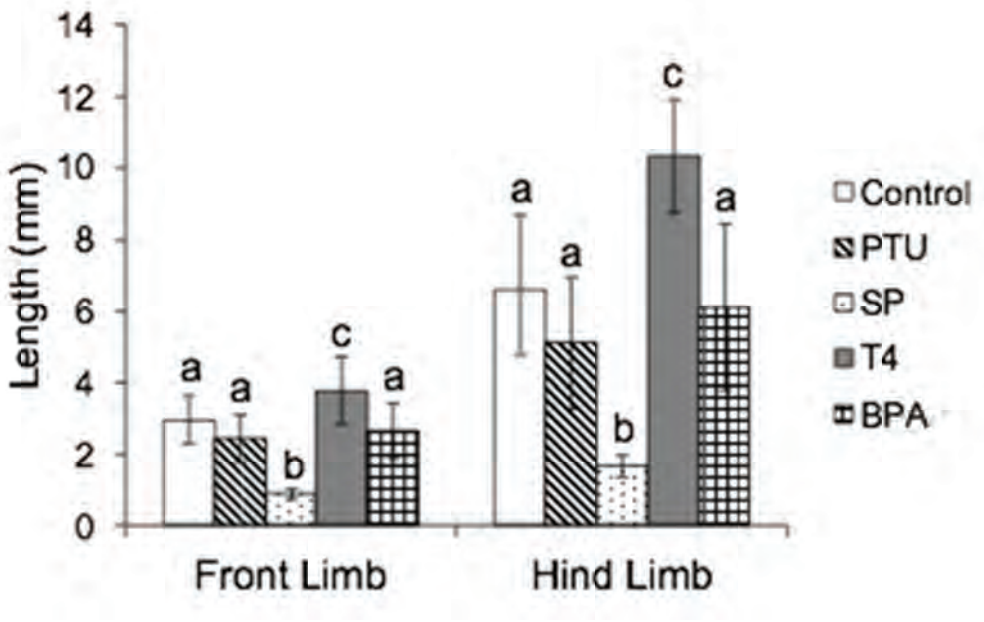

Figure 21: Mean ± standard deviation of the hind- and frontlimb lengths of *Xenopus laevis* tadpoles exposed for 21 days to buffered RO water (Control), propylthiouracil (PTU), sodium perchlorate (SP), thyroxine (T4) and bisphenol A (BPA) respectively. Dissimilar letters indicate statistically significant differences (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks).

There was a reasonable strong relationship (R^2 = 0.81) between NF stage and the increase in length of the hindlimb but more variation around the regression line with forelimb as independent variable (R^2 = 0.68). Whether the variation deviate from the expected (normal growth trajectory) as NF development progresses, needs more detail study (analysis of covariance, ANCOVA), including larger sample sizes.

3.3.1.4 Whole body length, Tail length and body mass variation

During the 7-day exposure, Snout to Vent length (SVL) and total body length (TBL) did not change significantly (Figure 22). In Figure 22, the average TBL of tadpoles in the perchlorate were significantly longer than those in the thyroxine group, although relative to the control, no significant (P>0.05) change in TBL can be reported. A similar trend was observed in tail length (TL) (Figure 22).

Snout to vent length and TBL did not vary much along the NF development gradient with the extension of the exposure period to 21 days (Figure 23). In contrast the TBL and SVL of the day-7 tadpoles, where some variation in these traits were obvious, in the 21-day exposure, TBL and TL only varied significantly in the T4 exposed tadpoles, i.e., they become shorter in body length and tail, marking the onset of apoptosis during metamorphosis climax (Figure 23).

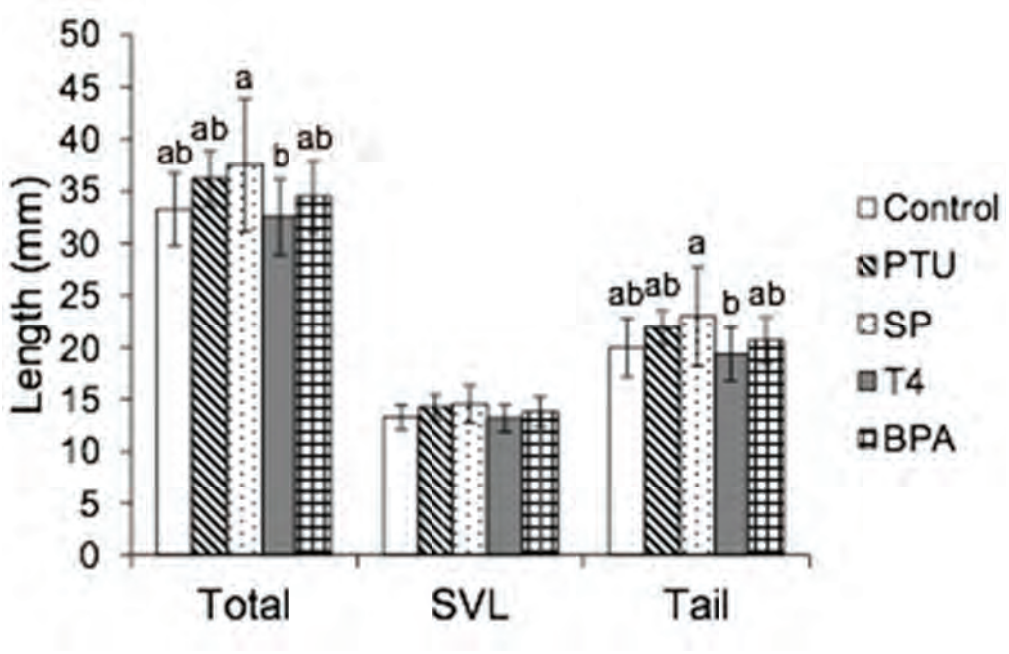

Figure 22. Mean ± standard deviation of the total-, tail- and snout-to-vent lengths of *Xenopus laevis* tadpoles exposed for <u>seven days</u> to buffered RO water (Control), propylthiouracil (PTU), sodium perchlorate (SP), thyroxine (T4) and bisphenol A (BPA) respectively. Dissimilar letters indicate statistically significant differences (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks).

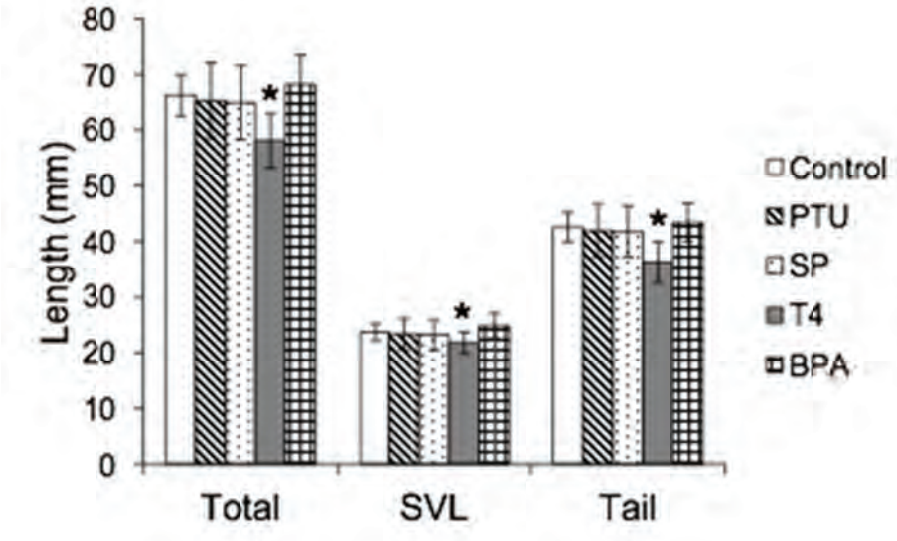

Figure 23. Mean ± standard deviation of the total-, tail- and snout-to-vent lengths of *X laevis* tadpoles exposed for <u>21 days</u> to buffered RO water (Control), propylthiouracil (PTU), sodium perchlorate (SP), thyroxine (T4) and bisphenol A (BPA) respectively. Asterisks (*) indicate statistically significant differences (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks).

Variation in body mass among the most of the exposure groups was not evident at the 7-day observation point (Figure 24). It was only the sodium perchlorate **(**SP) tadpoles that significantly gained weight (Figure 24). However, after the 21-day exposure, variation the body mass only occurred in the T4 group (significantly reduced) (Figure 25).

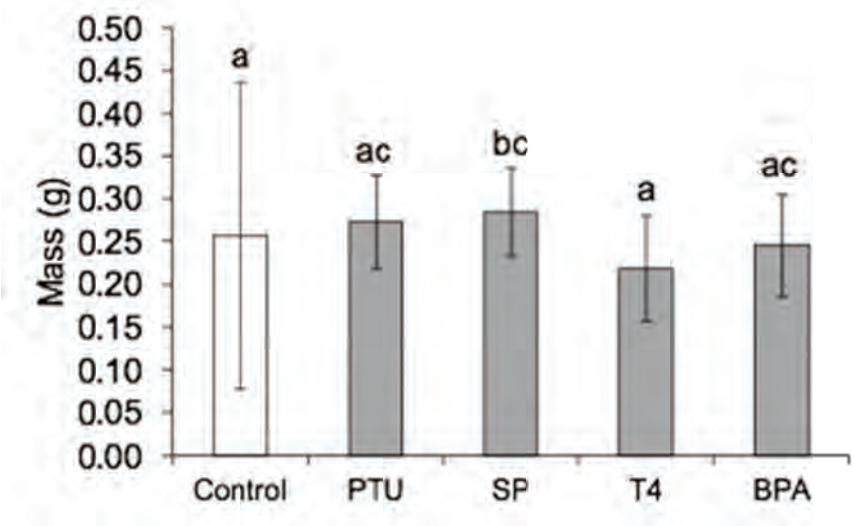

Figure 24. Mean ± standard deviation of the body masses observed for *Xenopus laevis* tadpoles exposed for <u>seven days</u> to buffered RO water (Control), propylthiouracil (PTU), sodium perchlorate (SP), thyroxine (T4) and bisphenol A (BPA) respectively. Dissimilar letters indicate statistically significant differences (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks).

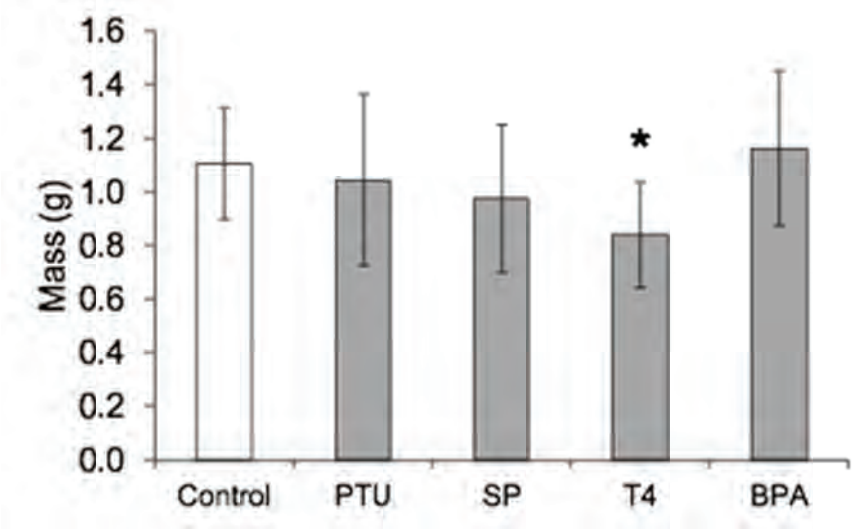

Figure 25: Mean ± standard deviation of the body masses observed for *Xenopus laevis* tadpoles exposed for <u>21 days</u> to buffered RO water (Control), propylthiouracil (PTU), sodium perchlorate (SP), thyroxine (T4) and bisphenol A (BPA) respectively. The asterisk (*) indicates a statistically significant difference (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks)

3.3.1.5 Histopathology

Histopathological changes in the thyroids collected from selected individuals (mostly the same NF-stage or a NF-stage close to median) were observed qualitatively as well as quantitatively. Both approaches suggested that significant histological changes were evident in the thyroids of the same stage tadpoles when compared among different exposure groups.

Control: – The tadpoles housed in the control water (buffered RO water) displayed healthy thyroids with minimum cellular hyperplasia (Figure 26; Table 6). Epithelial cells around the follicles were mostly cuboidal (Low) with little evidence of cellular hypertrophy (Figure 27). Colloid stained homogeneously, eosinophilic and displayed peripheral vacuoles. Colloidal (luminal) area was relatively high with very little inter-follicular tissue (Figures 26 & 27). From the histology it was clear that some hyperplasia may be visible (Figure 26), but this may be attributed to the growing thyroid associated with the developing tadpole. The thyroid histology/histopathology of tadpoles from exposure groups were compared to the control thyroid image.

PTU: – Tadpoles exposed to PTU did reach NF-stage 56 development (although the median was around NF-stage 55) as was the case in the Control group (Figures 18 & 19). Thyroid histology taken from the PTU exposed tadpoles clearly displayed a typical goiterous picture (enlarged gland area, Figure 27C) with an abundance of large follicles (Figures 27C). Follicular cells showed hyperplasia and were hypertrophic (Figure 26 & 27A,C, Table 6). Colloidal area was found to be significantly ($P<0.05$; Figure 27B) increased but the colloid in follicles displayed distended luminal cavities (partial colloid depletion) with hardly any peripheral vacuoles visible (Figure 26, PTU).

SP: – Similar to tadpoles exposed to PTU, tadpoles exposed to perchlorate (SP) showed decelerated development, not reaching NF-stage 56 (Control) (see Figures 18 & 19). For the histology investigation NF-stage 54 tadpoles were used. Total gland area was enlarged but not to the extent seen in tadpoles coming from the PTU tanks (Figures 26 & 27C). The follicular epithelium was extremely hyperplasic and hypertrophic and could not be measured as a simple epithelium (Figure 27A, Table 6). Characteristically, the colloidal (luminal) area in follicles were extremely reduced, epithelial infolding and follicular collapse present (Figures 26 & 27B).

T4: – Thyroxine exposed tadpoles showed significant ($P<0.05$) acceleration in development (Figures 18 & 19). The overall gland size at NF-stage 59 was significantly reduced (atrophic) (Figures 26 & 27B,C). Histologically, the T4 exposed tadpoles showed atrophic glands, with the colloidal area significantly reduced (Figure 27B).

Epithelial heights were significantly reduced (compared to that in the controls) but still relatively high with occasional hypertrophied cells (result of fast growth to metamorphosis) (Figure 27A, Table 6). Some indications of hyperplasia were also exhibited.

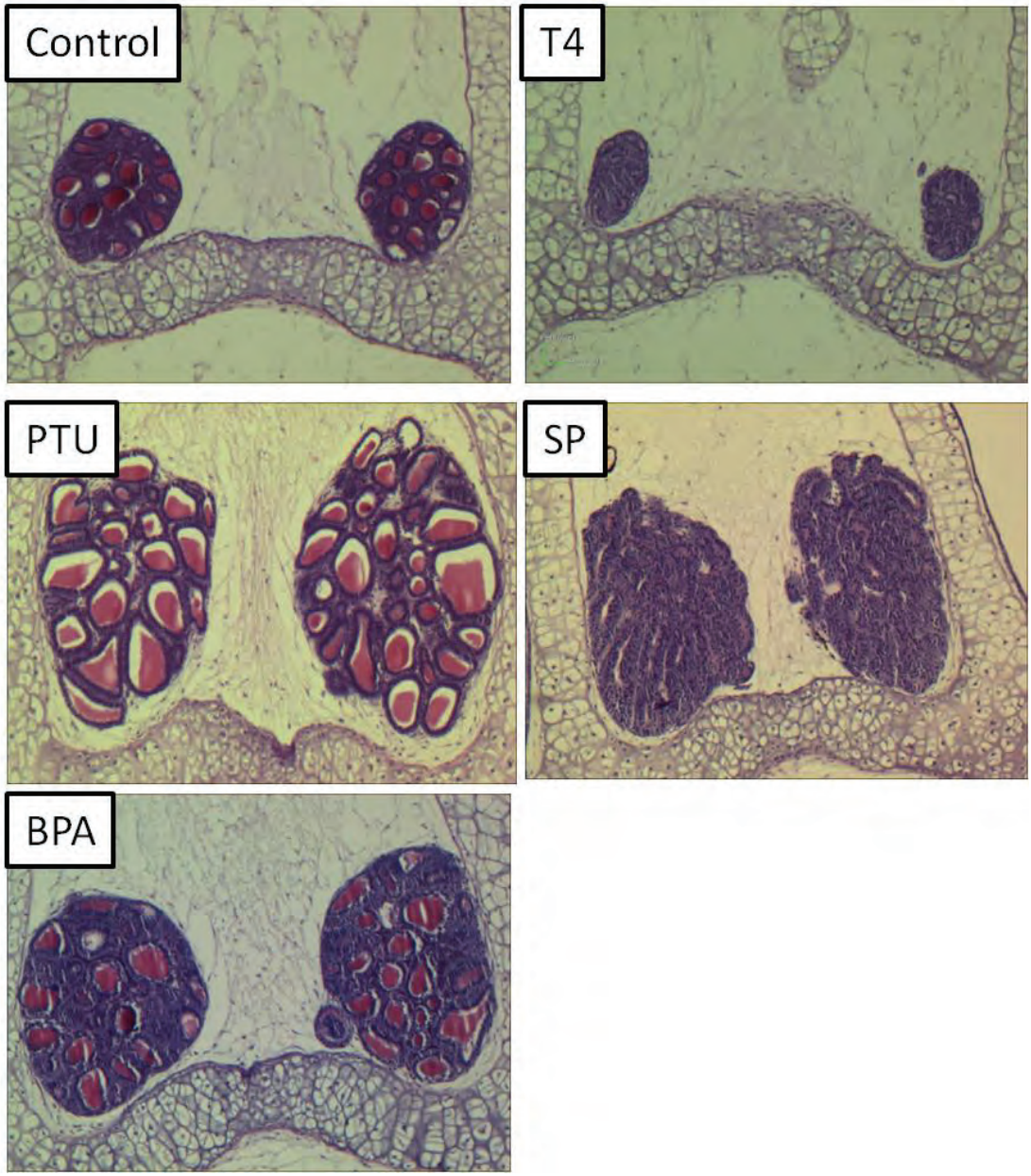

Figure 26. Qualitative changes in the thyroid histology of *Xenopus laevis* tadpoles after a 21-day exposure to a laboratory control (buffered RO water*) (*Control), thyroid hormone, thyroxin (T4), thyroid inhibitors, propylthiouracil (PTU), sodium perchlorate (SP) and bisphenol A (BPA). Sections were stained with Hematoxylin and Eosin (H&E) and light micrographs were taken at 20x magnification.

BPA: – Tadpole development was decelerated (but not significantly different from median NF stage 56 recorded for control tadpoles (Figure 18 & 19). Total thyroid gland area was significantly increased in BPA treated tadpoles (Figures 26 & 27C). Follicle epithelium height was increased (hypertrophic) and hyperplasia was visible (Figure 27, Table 6), both in epithelium and interfollicular region. Colloidal area was very similar to that of NF stage 56 tadpoles exposed to PTU (Figure 27B), although the extent of colloidal distend was not that extensive (but visible) (Figure 26).

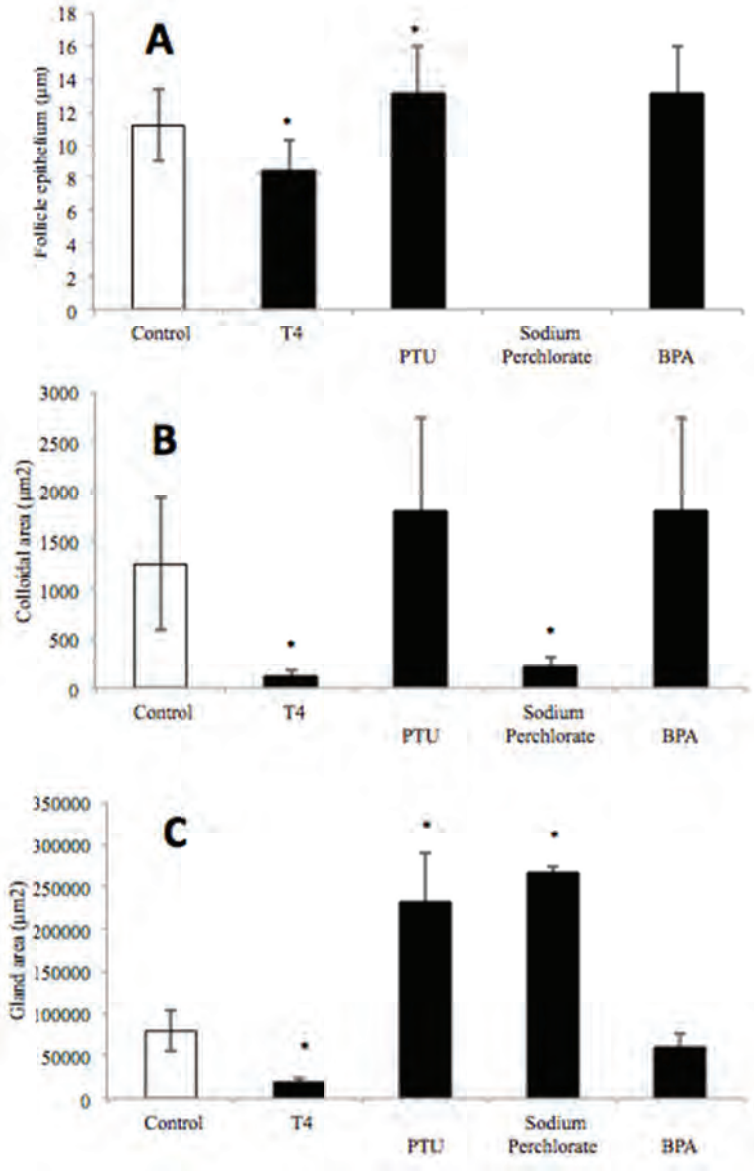

Figure 27. Histological measurement of thyroid traits: (A): Variation in mean follicular epithelium height among exposure groups, (B): Variation in colloidal area in follicles, (C) Variation in mean thyroid gland area. Axis labels correspond to the histological plates in Figure 26. Asterisk above bars indicate significance (P<0.05; Kruskal-Wallis test with Tukey's HSD multiple comparison test; Control vs. Experimental group).

Table 6. Treatment-related histopathology findings for the thyroid gland of *Xenopus laevis* tadpoles exposed to the validation compounds, Control, Thyroxin (T4), (PTU), Sodium Perchlorate (SP) and Bisphenol A (BPA) for 21 days. For each group a total of five tadpoles were studies/evaluated. The severity index scores (after Grim *et al.*, 2009) were, 0= not remarkable; 1=Mild; 2=Moderate; 3=Severe.

Histopathology	Severity Grade	Control	T4	PTU	SP	BPA
Thyroid Gland Atrophy	0	100	0	100	100	100
	1	0	70	0	0	0
	2	0	30	0	0	0
	3	0	0	0	0	0
Thyroid Gland Hypertrophy	0	100	100	0	0	40
	1	0	0	70	0	60
	2	0		30	70	0
	3	0	0	0	30	0
Follicular cell Hypertrophy	0	100	60	20	0	0
	1	0	40	50	0	30
	2	0	0	30	30	70
	3	0	0	0	0	0
Follicular cell Hyperplasia	0	100	0	40	0	0
	1	0	80	60	0	40
	2	0	20	0	0	60
	3	0	0	0	100	0
Histological Lesions	0	100	0	0	0	60
	1	0	60	20	0	40
	2	0	40	60	20	0
	3	0	0	20	80	0
N		5	5	5	5	5

3.3.2 Environmental water exposure studies

3.3.2.1 Kuils River – Eerste River Catchment (Semistatic exposure)

Variation in NF Developmental stages

Water samples (25 L) brought in from the field sites for exposure allowed for the first time for an assessment of potential thyroid disruption in water samples collected from river sites. The distribution of NF developmental stages for tadpoles exposed to RO water (buffered with marine salt) (Control) was normal with the NF developmental median at NF-stage 54. Tadpoles exposed to water from Site 1 (Bottelary River, upper tributary of the Kuils River) (Figure 12) over a period of 21 days showed and acceleration of development relative to tadpoles from the control tanks (Figure 28B). Similarly, water collected at most of the other downstream sites resulted in acceleration of development (Figure 28) with metamorphs (froglets) present in most tanks. Water from most of the in-stream sites resulted in a range of developmental stages (Figure 28).

Statistically most of the downstream sites (excluding site 3, Zandvliet WWTW) showed significant increases in developmental stage. Tadpoles exposed to Bottelary River (site 1) and the Kuils River downstream of the Belleville WWTP (site 2) showed the highest acceleration of development. Although median NF-stages of tadpoles exposed to water collected after the Kuils- and Eerste River confluence (sites 4 and 5) were lower, the median NF-stage was still higher compared to the tadpoles maintained in clean water (Figure 29).

Mean tadpole size varied significant among exposure groups and tadpoles from sites 1, 2, 4 and 5 were the largest (Figure 30). Tadpoles exposed to water collected downstream of Zandvliet WWTW (site 3) did not differ in size from the control tadpoles (although NF development was accelerated).

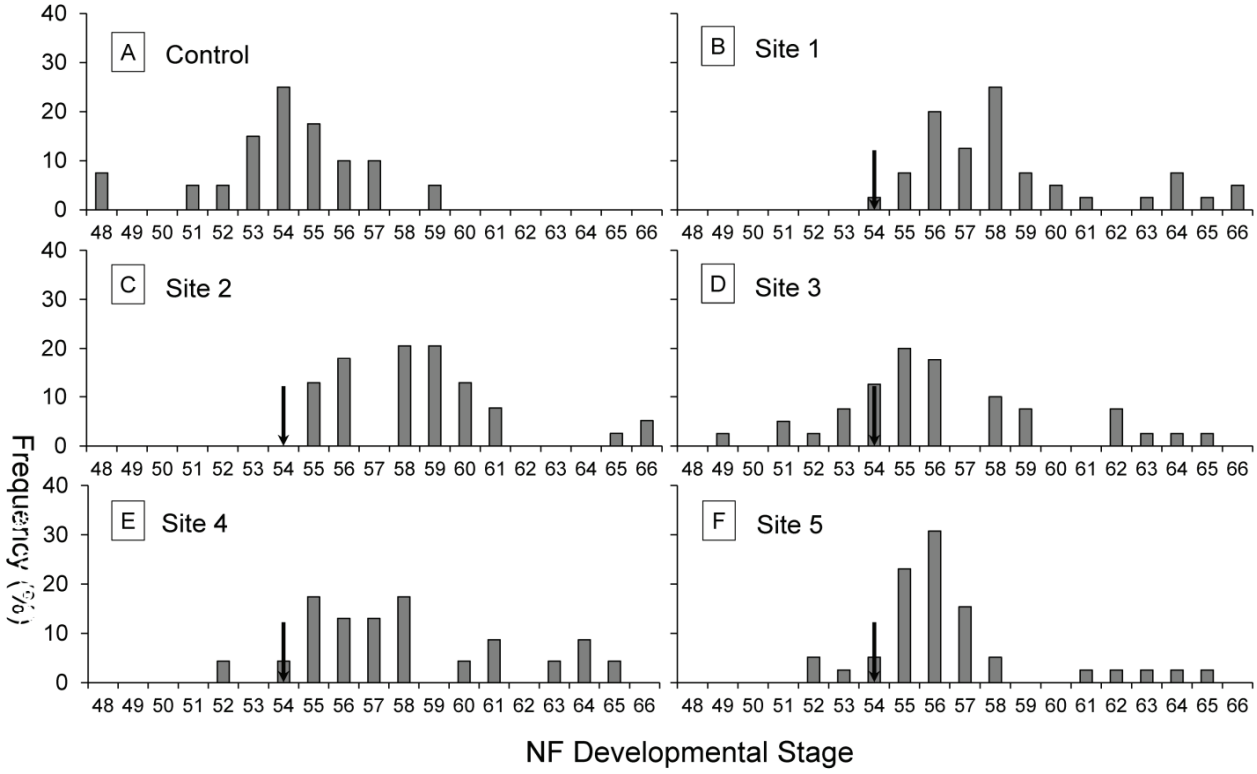

Figure 28: The frequency distributions of developmental stages (Nieuwkoop an d Faber 1958) attained by *Xenopus laevis* tadpoles exposed for 21 days to (A) buffered RO water (i.e., Control) as well as water collected from five localities (see Figure 12 & Table 4) within the Kuils River – Eerste Rivier catchment (B-F), Western Cape Province, South Africa. Vertical arrows indicate the median NF stage attained in the Control exposure group. Upstream, (1) Bottelary tributary, (2) Kuils River at N2, (3) Downstream of Zandvliet WWTW, (4) Eerste River before confluence, and (5) before mouth, downstream of Macassar WWTW.

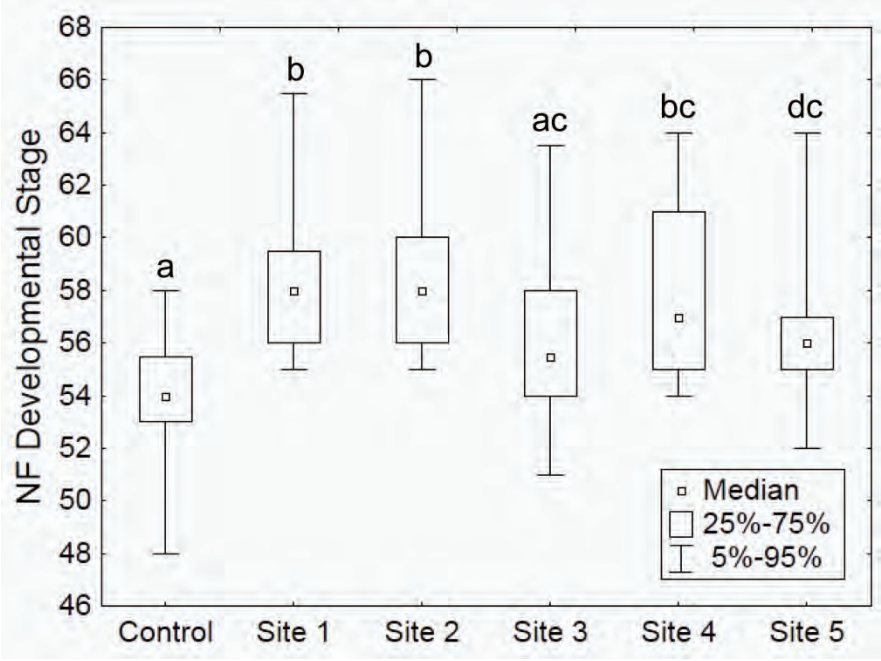

Figure 29. NF developmental stages observed in *Xenopus laevis* tadpoles exposed for 21 days to buffered RO water (i.e., Control) as well as water collected from five localities within the Kuils River – Eerste Rivier catchments, Western Cape Province, South Africa. Dissimilar letters indicate statistically significant differences (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks). For site information, see Figure 28 and Figure 12 and Table 4).

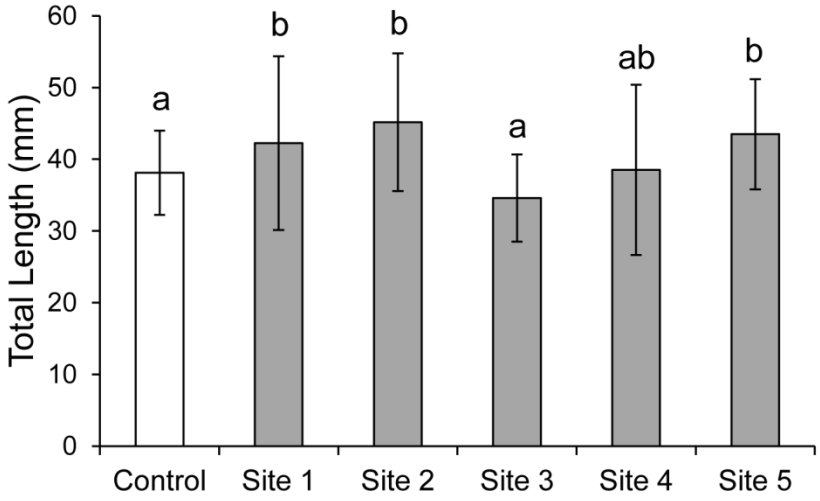

Figure 30. Mean ± standard deviation of the total lengths attained by *Xenopus laevis* tadpoles exposed for 21 days to buffered RO water (i.e. Control) as well as water collected from five localities within the Kuils River – Eerste River catchment, Western Cape Province, South Africa. Dissimilar letters indicate statistically significant differences (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks). For site information, see Figure 28 and Figure 12 and Table 4).

Histopathology

No histopathological analysis was conducted in this study.

3.3.2.2 City of Windhoek (Goreangap WWTP) (Semistatic exposure)

Variation in NF Developmental stages

Tadpoles exposed to the laboratory control water developed normally and median NF-stage for the group was NF-stage 56. No tank effects were observed and could the tadpoles from replicates be pooled. The only exception was the tadpoles in the Final sample (downstream of the Granular Activated Charcoal treatment point) (see Table 5). Tadpoles generally developed slowly (NF median at 55 vs. the 56 of Control group) and mortality was unacceptably high (50% died in the last two weeks of the assay). Apart from the Final samples, the tadpoles exposed to the other sites did not differ significantly ($P > 0.05$) in development rate when compared to the Control sample (Figures 31 & 32), around the median NF-stage of 56. Surviving tadpoles in the Final sample, although the distribution of NF stages was skewed to the left (Figure 31), the lower median NF developmental stage (NF-stage 55), proved not to be significantly different that of the Control tadpoles ($P>0.05$) (Figure 32). The Final sample was taken before the final blending with treated drinking water. Although the difference in NF stage development between the Final and Black Rose (blended domestic supply) samples differed slightly (not significantly different, $P>0.05$) the latter sample did not result in any significant difference compared to the Control or other sites.

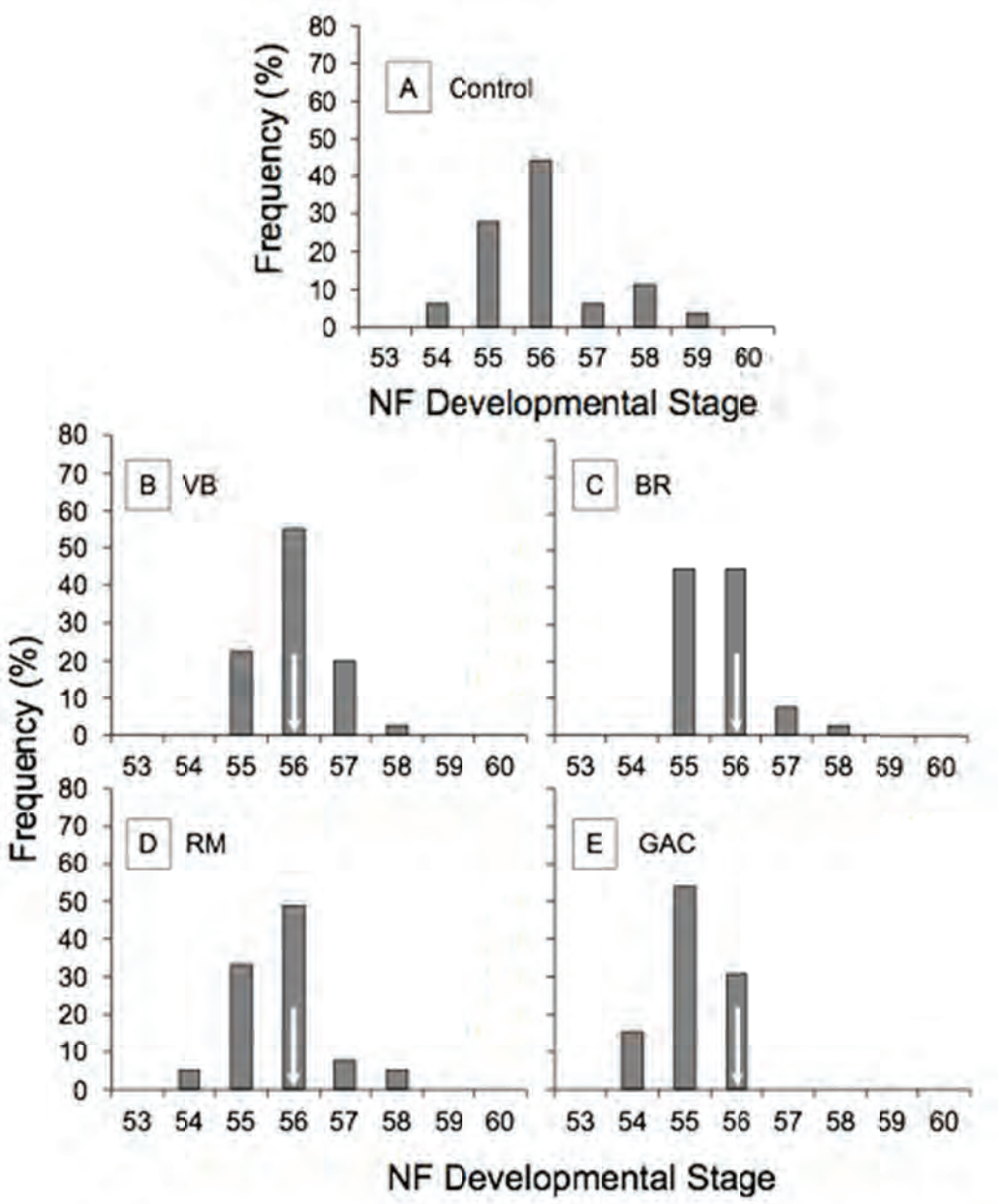

Figure 31: The frequency distributions of developmental stages (Nieuwkoop and Faber 1994) attained by *Xenopus laevis* tadpoles exposed for 21 days to (A) buffered RO H2O (i.e., Control), and water collected from (i.e., VB: Von Bach Dam (surface water inflow); BR: Black Rose (Residential Area); RM: Raw Mix (Goreangab dam and Gammas STP treated effluent); GAC (Final treated water, post granular activated carbon filter) within the Windhoek Drinking Water Treatment Operation, Goreangap plant, Namibia. Vertical arrows indicate the median stage attained in the control exposure group.

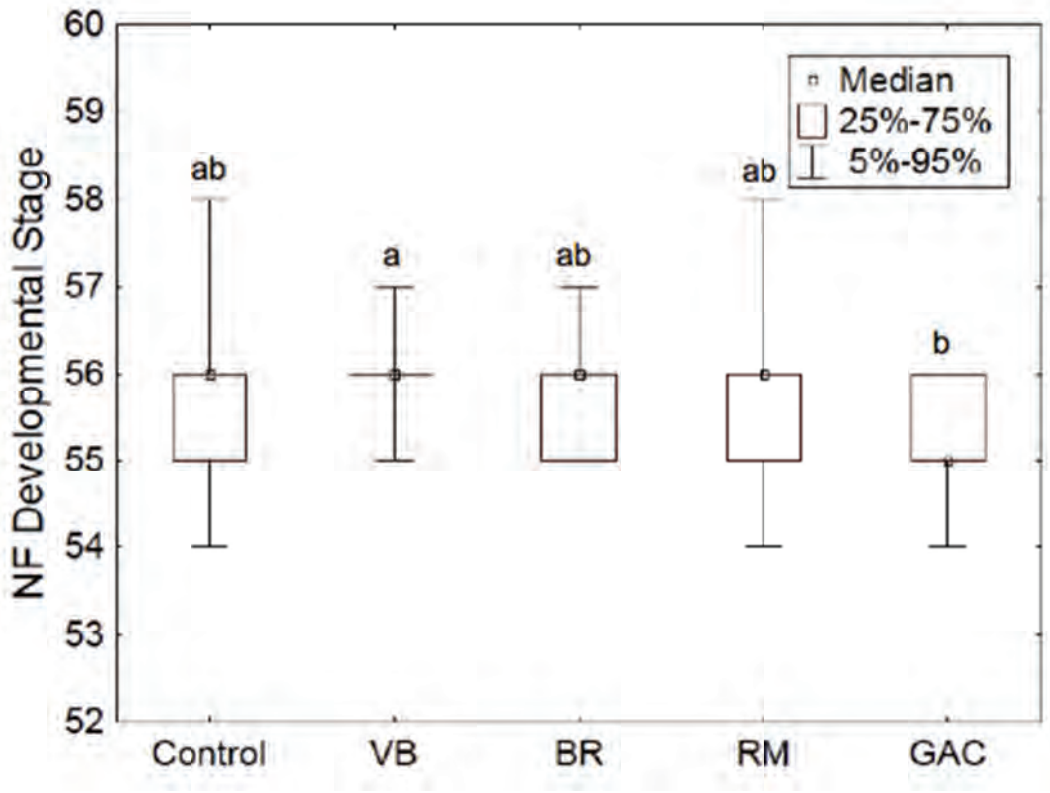

Figure 32. NF developmental stages observed in *Xenopus laevis* tadpoles exposed for 21 days to (A) buffered RO H2O (i.e., Control), and water collected from (i.e., VB: Von Bach Dam (surface water inflow); BR: Black Rose (Residential Area); RM: Raw Mix (Goreangab dam and Gammas STP treated effluent); GAC (Final treated water, post granular activated carbon filter, pre blending) within the Windhoek Drinking Water Treatment Operation, Goreangap, Namibia. Dissimilar letters indicate statistically significant differences (Kruskal-Wallis ANOVA, Multiple Comparisons of Mean Ranks).

Histopathology

For this study histopathological analysis was only done for the control exposures and the one site that showed some developmental effects, Final sample (downstream of the GAC filters). Histological sections presented in Figure 33A were included to investigate the possibility of some inhibition in the final, post-GAC sample. From the histology it seems that inhibition of TH has taken place and that thyroids display follicular cell hyperplasia and hypertrophy (Figure 33 &Table 7), a significant reduction in colloidal area (Figure 34B), and slight hypotrophy of the thyroid gland (Figure 35C). In general, the histology of these thyroid glands compare well to those seen in tadpoles exposed to inhibitors, like PTU and perchlorate (See Figures 26 & 27), although indications are that it represents a mild inhibitory response. Figure 33B, histological section of the thyroid of a Control tadpole, confirms the normal picture of colloid-filled follicles lined with simple cuboidal follicular cells exhibiting minimum follicular hyperplasia or hypertrophy.

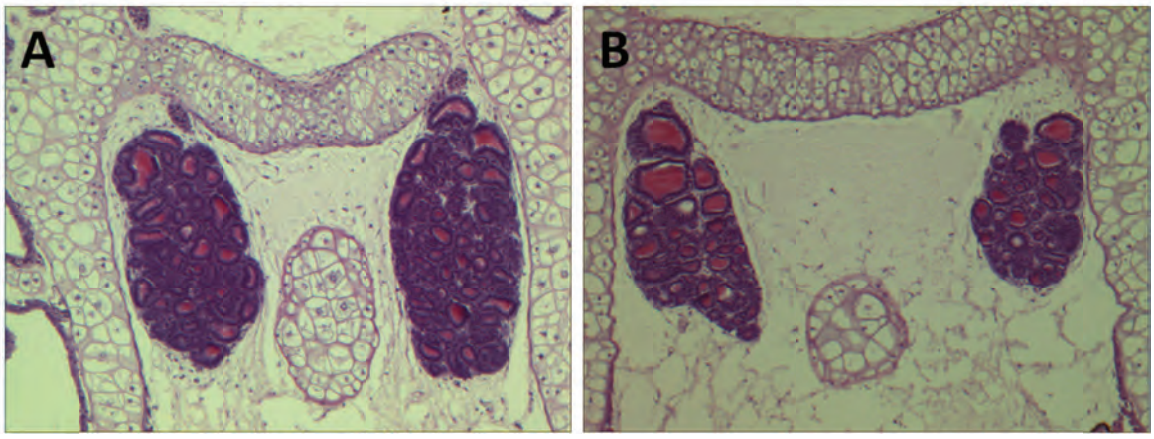

Figure 33. Thyroid histology of *Xenopus laevis* tadpoles during a 21-day exposure to water collected from the City of Windhoek, Goreangap treatment plant: A) Histology section of an example of a tadpole exposed to the Final sample (reclaimed and treated effluent after the Granular activated charcoal (GAC) filter, but before final mixture). The median NF stage development of tadpoles in this group was decelerated compared to tadpoles from the other groups (see Figures 31 & 32). B) Histology section of the thyroids of a tadpoles exposed to the laboratory Control medium (buffered RO water). Sections were stained with Hematoxylin and Eosin (H&E) and light micrographs were taken at 20x magnification.

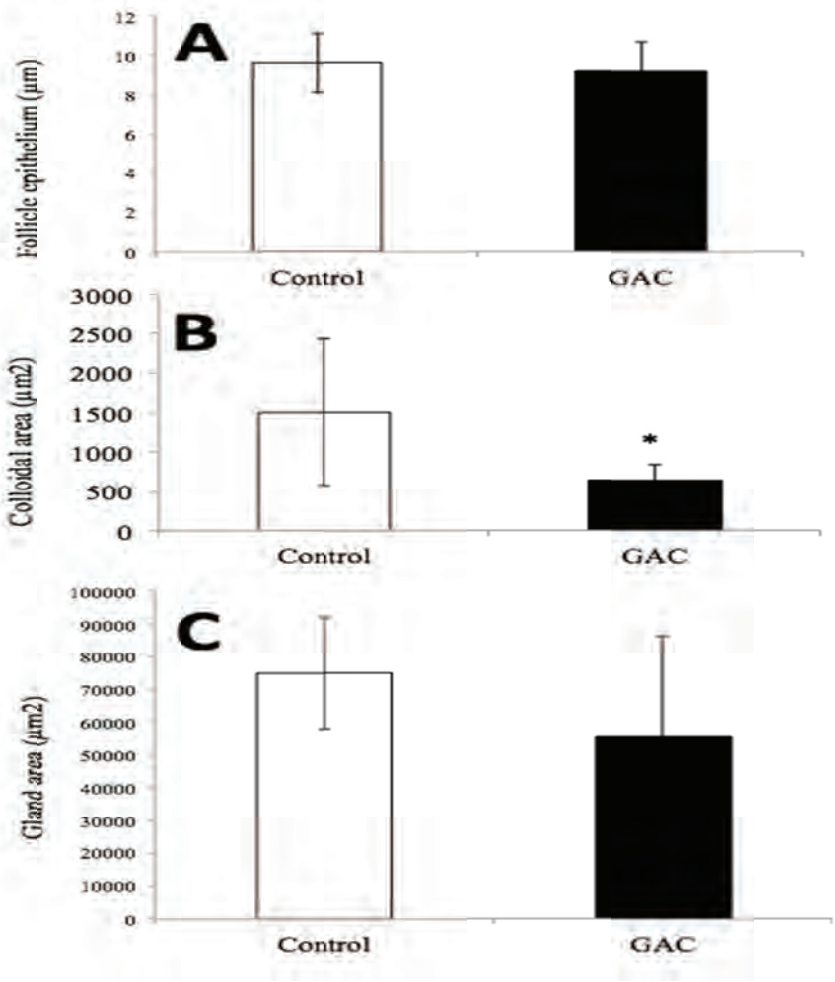

Figure 34. Histological measurement of thyroid traits: (A): Variation in mean follicular epithelium height, (B): Variation in colloidal area in follicles, and (C) Variation in mean thyroid gland area, among control and exposure groups. Axis labels correspond to the histological plates in Figure 34. Asteriks above bar indicate significance (P<0.05; Mann- Whitney U Test).

Table 7. Treatment-related histopathology findings for the thyroid gland of *Xenopus laevis* tadpoles exposed for 21 days to a Control water sample and a water sample collected from one site in the City of Windhoek, Goreangab reclamation plant during summer, November 2008. The sample was taken after the granular activated charcoal filter (GAC), final treated effluent. For each group a total of six tadpoles were selected for a histopathology evaluation. The severity index scores (after Grim *et al.,* 2009) were, 0= not remarkable; 1=Mild; 2=Moderate; 3=Severe.

Histopathology	Severity grade	Group	
		Control	GAC (Final)
Thyroid gland atrophy	Not remarkable	100	60
	Mild	0	40
	Moderate	0	0
	Severe	0	0
Thyroid gland hypertrophy	Not remarkable	100	80
	Mild	0	0
	Moderate	0	20
	Severe	0	0
Follicle cell hypertrophy	Not remarkable	100	80
	Mild	0	20
	Moderate	0	0
	Severe	0	0
Follicle cell hyperplasia	Not remarkable	100	100
	Mild	0	0
	Moderate	0	0
	Severe	0	0
	n	5	5

3.3.2.3 City of Cape Town (Zandvliet WWTP) (Flow through exposure)

Variation in NF Developmental stages

At the end of the 21-day exposure, the distribution of developmental stages in the laboratory Control group was normal with the median NF-stage being 57 (Figure 35). No mortality of tadpoles occurred in the Control or any of the exposure groups. The NF-stage distribution in all the other groups were similar, among replicates and the three groups (NF median stage being 56 for both 50% and 100% effluent groups) did not vary significantly among exposure groups ($P>0.05$) (Figure 36). The median developmental stage being NF-stage 57.

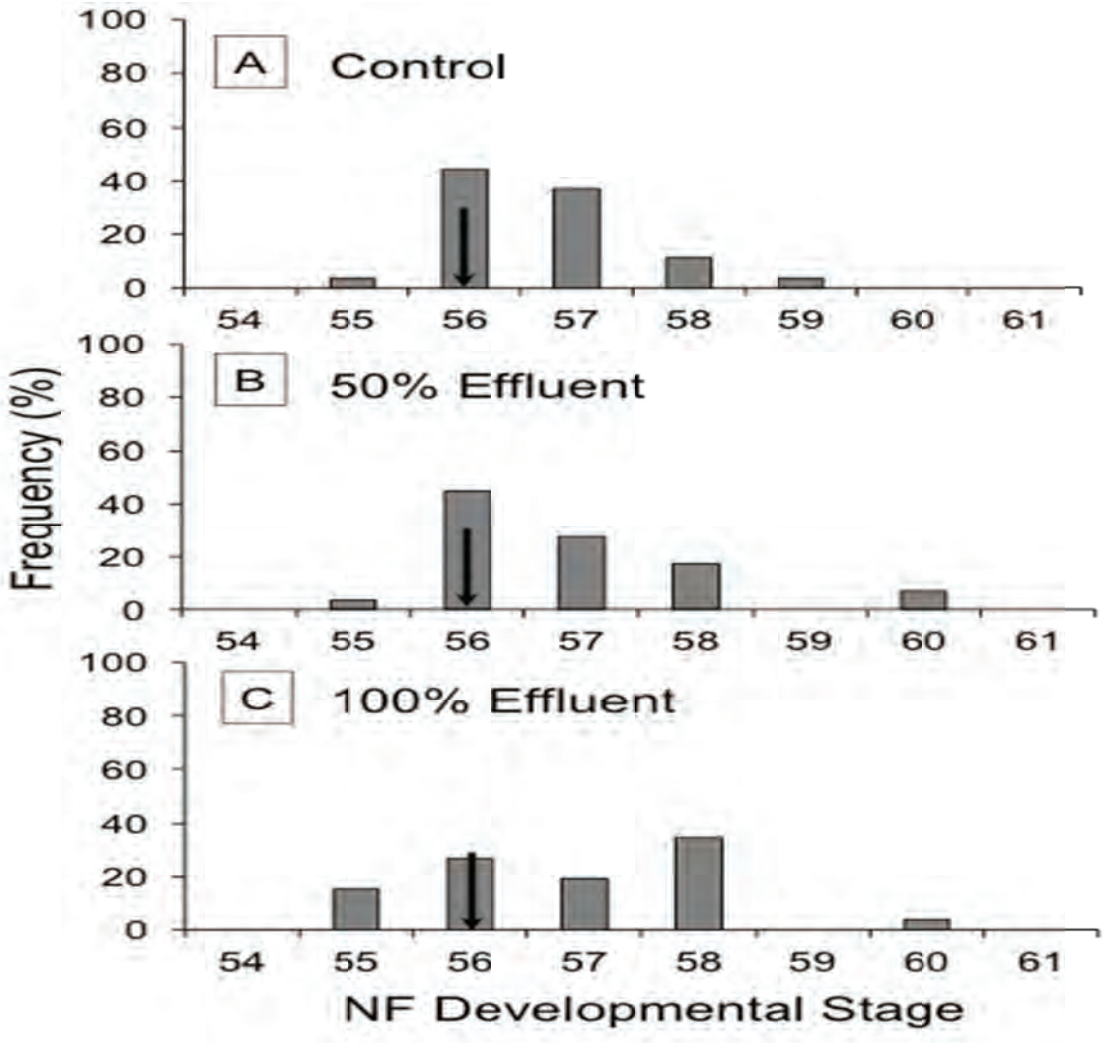

Figure 35: The frequency distributions of developmental stages (Nieuwkoop and Faber 1956) attained by *Xenopus laevis* tadpoles exposed for 21 days to (A) buffered RO water, (B) 50% diluted WWTP effluent and (C) pure WWTP effluent collected from the Zandvliet treatment works, Cape Town, South Africa. Vertical arrows indicate the median NF stage attained in the Control exposure group.

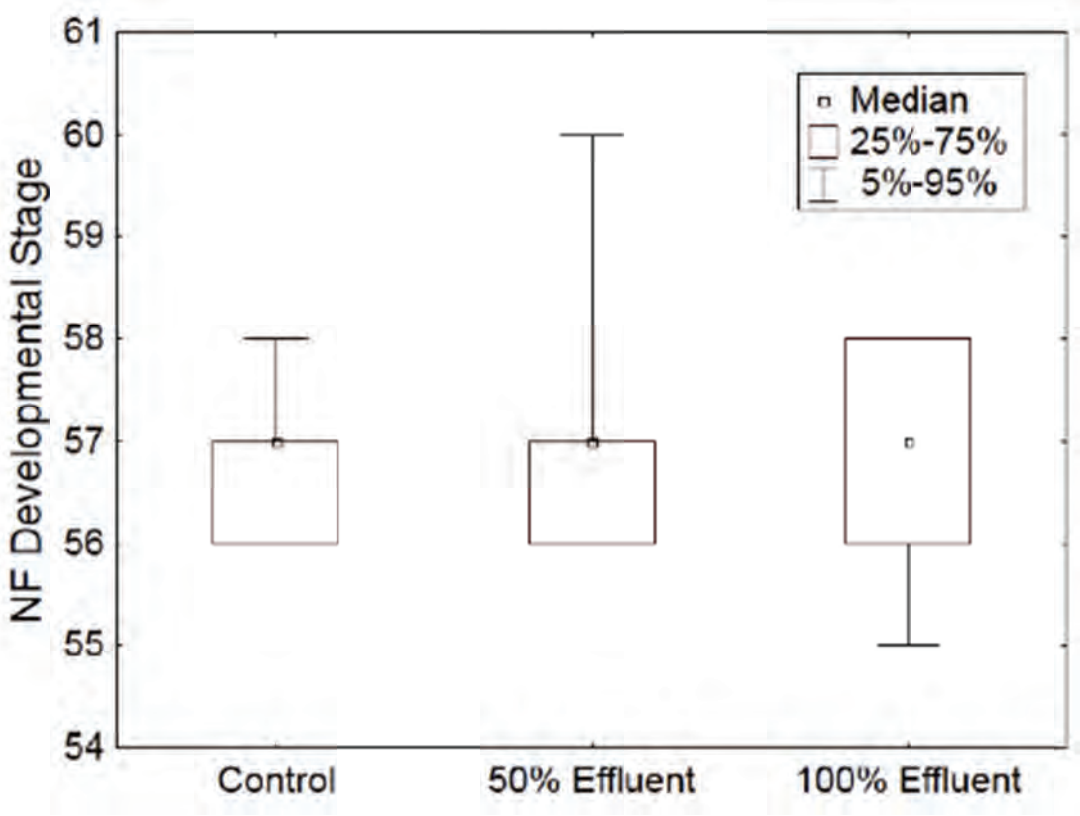

Figure 36. NF developmental stages observed in *Xenopus laevis* tadpoles exposed for 21 days to buffered RO water, 50% diluted WWTP effluent and pure WWTP effluent collected from the Zandvliet treatment works, Cape Town, South Africa.

Variation in morphometrics and body size

Statistically no differences (P>0.05) were found in the median NF-stage among the exposure groups (Figure 36). Hindlimb and forelimb development also did not vary significantly among exposure groups (P>0.05) (Figure 37). Tadpoles in all groups were similar in body morphometrics, total length, SVL and tail length (Figure 38) as well as body mass (Figure 39).

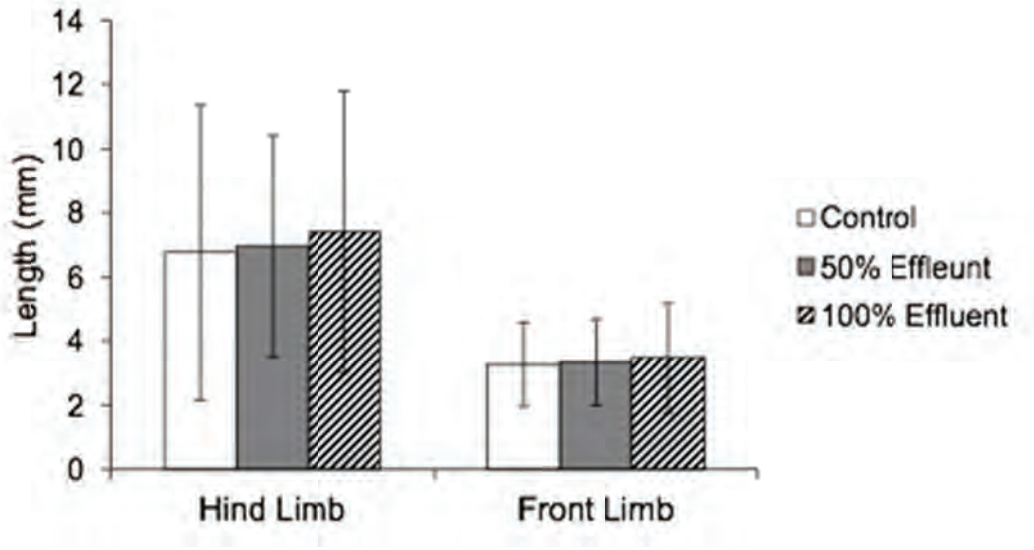

Figure 37: Mean ± standard deviation of the hind– and forelimb lengths of *Xenopus laevis* tadpoles exposed for 21 days to buffered RO water, 50% diluted WWTP effluent and pure WWTP effluent collected from the Zandvliet treatment works, Cape Town, South Africa.

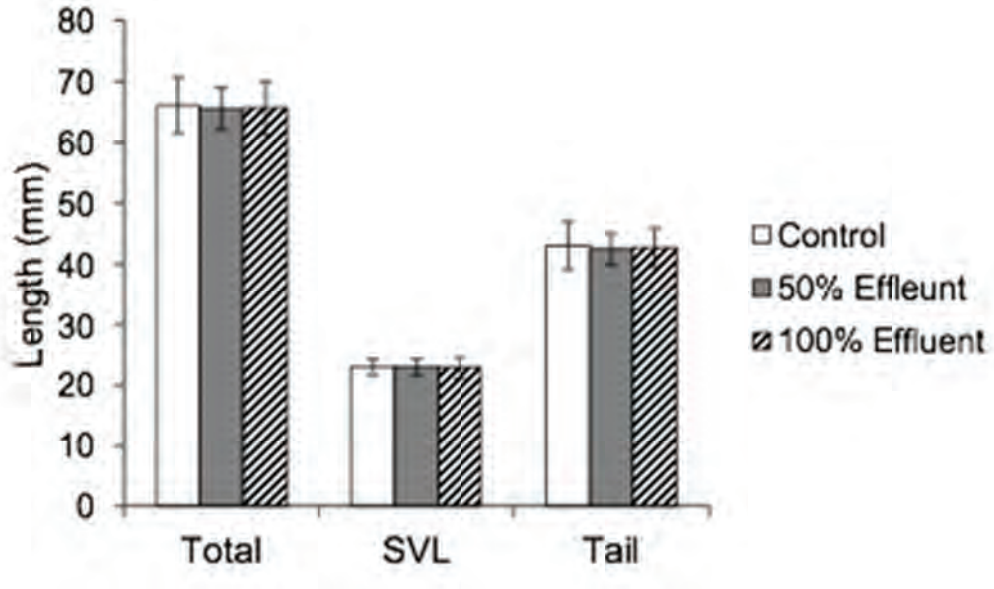

Figure 38: Mean ± standard deviation of the total-, tail- and snout-to-vent lengths of *Xenopus laevis* tadpoles exposed for 21 days to buffered RO water, 50% diluted WWTP effluent and pure WWTP effluent collected from the Zandvliet treatment works, Cape Town, South Africa.

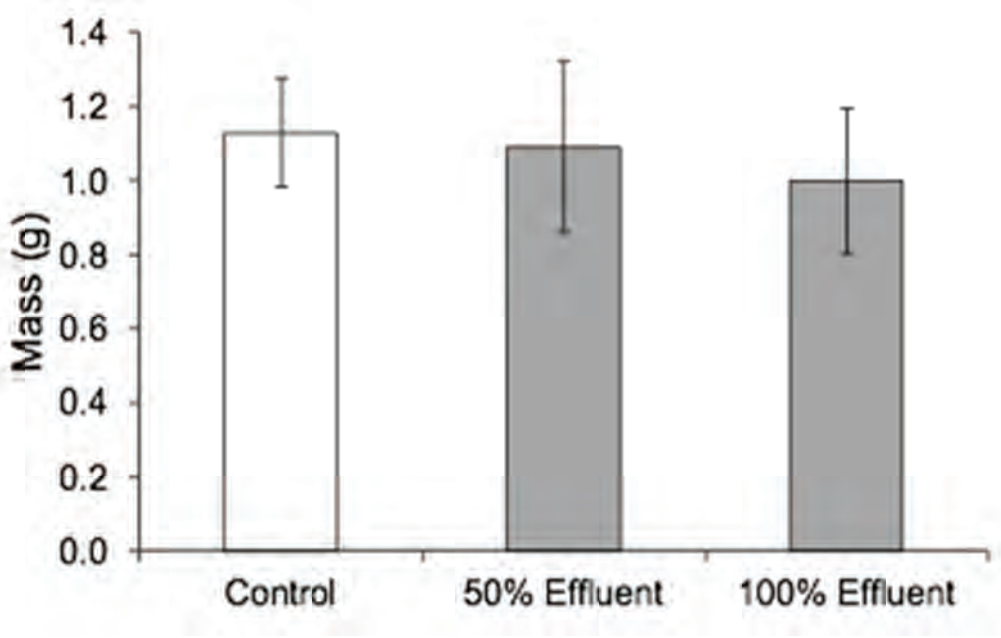

Figure 39: Mean ± standard deviation of the body masses observed for *Xenopus laevis* tadpoles exposed for 21 days to buffered RO water, 50% diluted WWTP effluent and pure WWTP effluent collected from the Zandvliet treatment works, Cape Town, South Africa.

Histopathology

In terms of NF stage variation, no significant change in NF- stage occurred during the 21-day exposure to Zandvliet final effluent, median around NF-stage 56 (Figures 36). Histologically, the general appearance of the thyroid glands of the control and undiluted effluent samples was very similar (Figure 40). However, the total gland area was significantly increased ($P<0.05$) in the effluent (100% sample) exposed tadpoles (Figure 41C), but the colloidal/luminal areas did not differ significantly ($P>0.05$) (Figure 41B) and the average follicle epithelial cell height was similar in the two groups (Figure 41A). Although the increase in size of the gland in undiluted effluent exposed tadpoles may suggest indications of thyroid inhibition (similar to PTU), the general histological appearance of the glands did not show marked differences between the two groups. The thyroid gland area as a measure of the size of the gland in NF-56 stage tadpoles did change significantly when compared to the control-tadpoles (Figure 41C). Some hyperplasia could be seen in the effluent exposed tadpoles but this could be normal growth related in these developing tadpoles (more tadpoles will be sectioned) (Figure 40). Mild follicular epithelium hypertrophy and hyperplasia was observed in the 50% diluted effluent exposure group (Table 8), and this lead to the significant differences in follicle epithelium height when comparing the thyroids

from tadpoles taken from the two effluent groups (Figure 41A). However, the increase in epithelial height did not vary significant from the control.

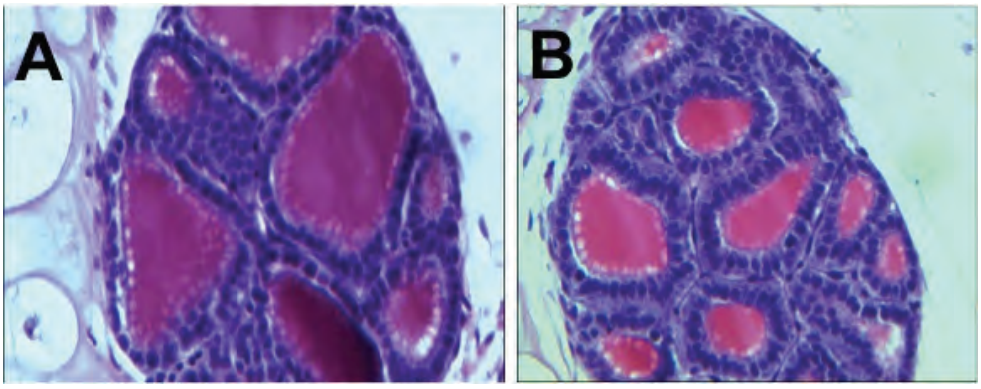

Figure 40: Light micrographs of sections of the thyroid of *X. laevis* NF stage 56 tadpoles (21 day exposure) that was exposed to (A) Control water (buffered RO water) and (B) undiluted (100%) final, treated effluent taken from the City of Cape Town, Zandvliet WWTP.

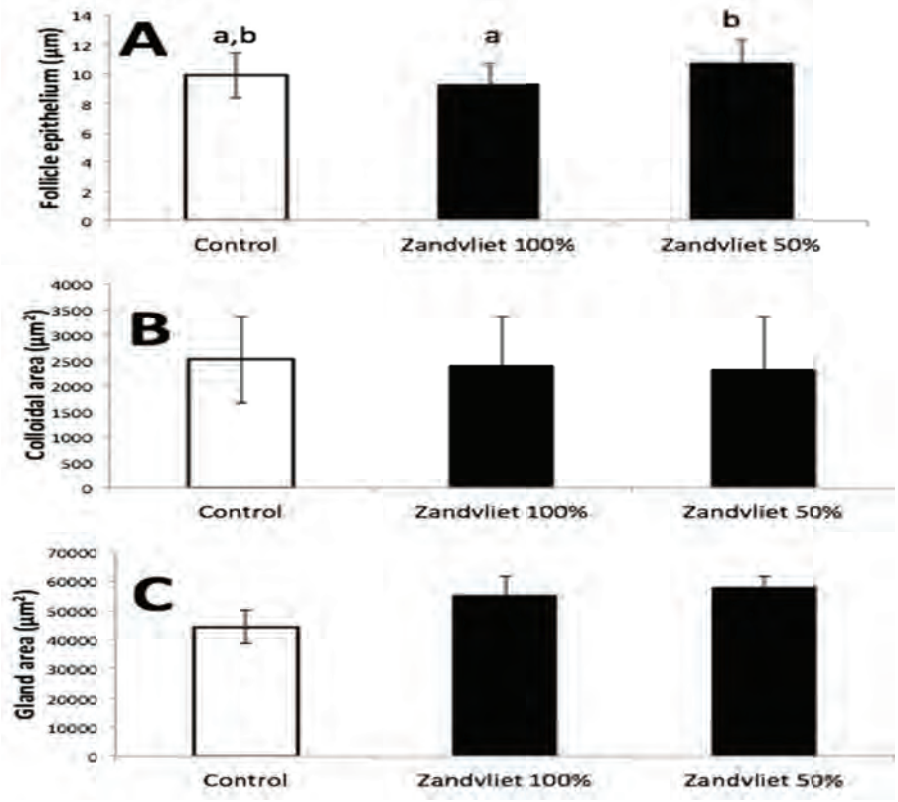

Figure 41. Histological measurement of thyroid traits of *Xenopus laevis* tadpoles exposed to final treated effluent water from Zandvliet WWTP close to the City of Cape Town: (A): Variation in mean follicular epithelium height among exposure groups, (B):Variation in colloidal area in follicles, (C) Variation in mean thyroid gland area. Significant variation among groups were only found in the epithelium height endpoint ($P<0.05$; ANOVA, with Tukey's HSD multiple comparison test (Control vs. exposure group). Dissimilar letters indicate statistically significant differences.

Table 8. Treatment-related histopathology findings for the thyroid gland of *Xenopus laevis* tadpoles exposed for 21 days in a flow-through system to a Control water sample and final treated effluent (undiluted (100%) and diluted (50%) collected from Zandvliet WWTP close to the City of Cape Town. For each group a total of five tadpoles were subjected to histology procedures to evaluate the histopathology. The severity index scores (after Grim *et al.,* 2009) were, 0= not remarkable; 1=Mild; 2=Moderate; 3=Severe.

Histopathology	Severity grade	Groups		
		Control	50% Final Effluent	100% Final Effluent
Thyroid gland atrophy	Not remarkable	100	100	100
	Mild	0	0	0
	Moderate	0	0	0
	Severe	0	0	0
Thyroid gland hypertrophy	Not remarkable	100	60	100
	Mild	0	40	0
	Moderate	0	0	0
	Severe	0	0	0
Follicle cell hypertrophy	Not remarkable	100	60	100
	Mild	0	40	0
	Moderate	0	0	0
	Severe	0	0	0
Follicle cell hyperplasia	Not remarkable	100	80	100
	Mild	0	20	0
	Moderate	0	0	0
	Severe	0	0	0
	n	5	5	5

3.4 Discussion

Laboratory validation of XEMA

The aim of the present study was to use the methodological guidelines for the standardized XEMA set-up to screen for potential thyroid disruptors and conduct controlled laboratory exposures to evaluate the application of this bioassay approach, in South Africa. Although the underlining rationale of using amphibian metamorphosis to detect the presence of environmental thyroid disruptors through alterations in the normal developmental program, known to be dependent on the normal function of the thyroid system (Shi, 1999), it was only recently that the XEMA set-up underwent extensive inter-laboratory validation (Degitz *et al.,* 2005; Opitz *et al.,* 2005; Coady *et al.,* 2010). The XEMA assay was designed around the assumption that the disruption of the thyroid system in some way or another will be reflected in the developmental rates of the tadpoles. Therefore, compounds antagonizing the actions of TH will slow down developmental rates and substances that mimic or enhance the actions of TH would accelerate the development towards metamorphosis climax. Initially the assay was designed around the role of TH in tail resorption (Fort *et al.,* 2000). Important endpoints used initially included tail length and in some cases time to metamorphosis, but in the current XEMA it is the NF developmental stage (NF) that forms the primary endpoint (Opitz *et al.,* 2005). General growth endpoints as well as morphometric endpoints, including tail length, total length and body mass are still included (monitored) since modulation of tadpole development (including non-specific toxic effects) may be mirrored in other general growth endpoints. As more knowledge became available regarding the expression of receptors of TH in target tissues, the development of the limbs (high expression of TH receptors) were included as endpoints (Degitz *et al.,* 2003; Opitz *et al.,* 2006b). More recently, histopathological aspects of the thyroid itself (Grim *et al.,* 2009) were included and expression of specific target genes, specifically the thyroid hormone receptor mRNAs (TRα and TRβ) (Crump *et al.,* 2002; Veldhoen *et al.,* 2002; Jagnytsch *et al.,* 2006; Opitz and Kloas, 2010).

The premetamorphic phase leading on to the prometamorphic phase was recognized as the most important window to conduct the XEMA in (Kloas, 2002; Touart, 2002; Degitz *et al.,* 2005; Opitz *et al.,* 2005). During this period the expression of the TH receptor (TRβ) in target tissues and the endogenous concentrations of TH are low (Shi, 1999), but the potential of receptor up-regulation and acceleration of development upon precocious induction by exogenous TH already exists in premetamorphic tadpoles. Chemicals with thyromimetic activities would then be exposed during exposure to premetamorphic tadpoles (around NF stage 51). On the other hand, compounds with antithyroidal activities would delay the onset of thyroid-mediated development during the prometamorphic phase and

eventually also lead to delays in metamorphosis. In most XEMA set-ups, especially when screening individual chemicals, control chemicals, Thyroxine and/or an inhibitors of T4 production, PTU are included as positive and negative controls respectively. The lack of sensitivity to thyromimetic activity when using the tail resorption assay was attribute to the relative high endogenous TH at the onset of metamorphosis (Kloas *et al.,* 2002). The same explanation holds for the superior sensitivity regarding the up-regulation of specific TH-responsive genes (Opitz *et al.,* 2006b; Opitz and Kloas, 2010). When screening environmental water it may not be necessary to include positive and negative control groups when conducting a XEMA. In such cases agonistic or antagonistic activity in site-samples are compared to a laboratory negative Control sample. However, the inclusion of chemicals as control groups increases the validity of the testing procedure.

The T4 concentration selected was taken from the validation studies conducted (Opitz *et al.,* 2005; Tietge *et al.,* 2005; Grim *et al.,* 2009) and was nontoxic to tadpoles and produced a significant acceleration of metamorphosis. The present study also confirmed that the premetamorphosis show adequate sensitivity to be used to identify thyromimetic activity of potential EDCs. In most early studies regarding XEMA, propylthiouracil (PTU) was included as a positive control for antithyroidal activity (Tietge *et al.,* 2005; OECD, 2007c; Opitz *et al.,* 2009; Tietge *et al.,* 2010). Following the application of XEMA in studies regarding perchlorate salts (Carr *et al.,* 2008), perchlorate (SP, PER) has been included as anti-thyroidal control. Although inhibition of the thyroid was achieved using both compounds, and TSH over-stimulation leading on to goiter formation, it was recently reported that at the molecular level variation in thyroid-related endpoints may be evident and be related to the anti-thyroid compound used (Tietge *et al.,* 2005; Opitz *et al.,* 2009; Tietge *et al.,* 2010). Most TH synthesis inhibitors will result in thyroid follicular cell hyperplasia and hypertrophy, alterations in the colloid and glandular growth, as part of a compensatory activity modulate by increasing circulating TSH concentrations in response to declining TH concentration in the blood (Tietge *et al.,* 2010). Propylthiouracil (PTU) specifically inhibits the synthesis of T4 in the thyroid by activation of thyroid peroxidase (Brucker-Davis, 1998). Inhibition metamorphic development mostly occurs soon after treatment commences. In the present *in vivo* study no significant change in median NF stage was observed after seven days of exposure and significant inhibitory effects could only be recorded after a longer exposure period (21 days). The hindlimbs were significant larger than controls and similar to the length of hindlimbs of tadpoles exposed to sodium perchlorate. Perchlorate as inhibitory control in the present study resulted in stronger inhibition (using NF stage as criterion). The mechanism of action of perchlorate is different, i.e., responsible for inhibiting iodide uptake (inhibiting the sodium-iodide symporter pathway) into the follicular cells (Wolff, 1998). It has

been pointed out that the different mechanism of actions that TH inhibitors may have could explain the different sensitivities among inhibitors (Opitz *et al.,* 2009). In light of the low mortality rates and effectiveness, the inclusion of positive and negative control-chemical groups to detect and characterize both stimulatory and inhibiting effects on the thyroid system in amphibians is preferable (if practical).

Bisphenol A (BPA) was included in this study because several reports suggested it to be a potential thyroid modulator (potentially could interfere with the action of TH at several levels) (Heimeier *et al.,* 2009; Heimeier and Shi, 2010a). This compound is currently recognized as one of the high volumes produced chemicals on a global scale and is associated with the production of plastics, polycarbonate plastics, epoxy resins as coating in food cans and also in dental sealants. Several reports have suggested that BPA may leach from plastic products and end up in the environment, animal bodies and human bodies (Heimeier and Shi, 2010a). Although BPA has been linked to feminization effects *in vivo*, the potential of BPA being involved in thyroid disruption could not be excluded and studied by several from this perspective (Iwamuro *et al.,* 2003; Zoeller *et al.,* 2005; Heimeier and Shi, 2010). The potential of BPA as TH antagonist have recently been investigated by several groups (Iwamuro *et al.,* 2003; Zoeller *et al.,* 2005; Heimeier and Shi, 2010). Heimeier *et al.,* (2009) did report no noticeable morphological changes after a treatment with BPA alone for a testing period of four days in *Xenopus* tadpoles. However, in combination with T3, the stimulatory effects associated with T3 were inhibited. In addition, Heimeier *et al.,* (2009) reported interference with the T3 moderated remodelling of the intestinal region. Studies in other anurans showed that BPA could block thyroxine induced or natural metamorphosis (Iwamuro *et al.,* 2003; Iwamuro *et al.,* 2006). Several of these studies showed stage and morphological change even after a short exposure period of 3-4 days (Iwamuro *et al.,* 2003; Heimeier *et al.,* 2009). In the present study, premetamorphic *X. laevis* tadpoles were exposed for 21 days to BPA and although there was some indication of a deceleration of the developmental progression as compared to the control group, the median NF stage did not differ significantly from that of the control tadpoles. As suggested before, it indications are that BPA on its own does not affect the TH controlled developmental program dramatically, but in the presence of TH (T3) it may well inhibit the actions/signalling of TH (as much as 30%) (Heimeier and Shi, 2010).

In the present study, additional morphological endpoints were included, i.e., body mass, total length, tail length and snout to vent length as well as the lengths of the fore- and hindlimbs. It is now well known that during metamorphosis, TH may be involved in the resorption of the tail. In spite of the tail being a response target for TH the first studies reported a rather low sensitivity for exogenous TH mimics or antagonists. In the literature, it

has been the topic of speculation whether this phenomenon (low sensitivity to exogenous TH) may be attributed to the presence of endogenous TH that could mask modulation by thyroid disrupting compounds. This was the initial basis from where the first XEMA was designed. Although it may be true that growth, in general (whole body length), will be affected by inhibitory effects that compounds may have on tadpole development, it was found that WBL may be an usable endpoint in a pre- and pro-metamorphosis type assays, but closer and during natural metamorphosis, the body undergo remodelling, i.e., natural shortening of the body and will it be difficult to differentiate causal effects. The continued use of WBL in XEMA seems to be rather valuable to assess toxic side effects on growth during the exposure period rather than being valuable as endpoint for thyroid disruption (Jagnytsch *et al.,* 2006; Coady *et al.,* 2010).

Molecular studies (temporal and spatial expression of TH response genes) showed that the developing limbs might be targets for TH action (Shi, 1999; Veldhoen and Helbing, 2001; Helbing *et al.,* 2003; Opitz *et al.,* 2006a; Helbing *et al.,* 2011). In an extensive study of differential expression of both TRα and TRβ, either during the spontaneous metamorphosis and TH-induced metamorphosis, Opitz *et al.,* (2006) confirmed the TH-induced upregulation of TRβ in several tissues investigated. During spontaneous metamorphosis Opitz *et al.,* (2006) showed the upregulation of TRβ in hindlimb tissue starting from NF- stage 55 through NF-stages 58/59 when TRβ expression peaked (late prometamorphosis), but then decreased toward peak of metamorphosis. Hindlimb morphogenesis, therefore, represents an early TH-regulated metamorphic event with TRβ expression that increase (between NF-stages 55 and 58) as limb growth and development progresses. Although not clear from the present study, it was reported that hindlimb might be a sensitive endpoint that showed significant increase within a 7-day period following TH induction (Mitsui *et al.,* 2006; Opitz and Kloas, 2010). But, during the 21-day XEMA, the thyroxine (T4) exposed tadpoles showed advanced development with increased hindlimb and forelimb lengths. On the other hand the tadpoles exposed to both TH inhibitory control compounds, PTU and perchlorate, showed decelerated development exhibiting smaller sized hind- and forelimbs. This response pattern is similar to the published XEMA validation reports (Opitz *et al.,* 2005; Jagnytsch *et al.,* 2006; Opitz *et al.,* 2006; Coady *et al.,* 2010). Opitz *et al.,* (2006) made an important point regarding the correlation between plasma TH levels and expression of TRβ, suggesting that although circulating TH plasma levels are an important factor determining TRβ expression, it may not be the sole factor controlling the level of expression. Clearly, this phenomenon needs more study and the high expression rate of TRβ in the hindlimbs make these candidates target tissue to study in future.

Histological/histopathological response to the control chemicals used in the XEMA set-up largely corresponded to the published validation and testing reports. The Control tadpoles generally exhibited normal histology, expected for a NF-stage 65 *Xenopus* laevis tadpole (Grim *et al.,* 2009). Although hyperplasia and hyperthrophy may not be the norm, it may be present because tadpoles used in the XEMA programme are growing and thyroid development continue through the 21-day exposure, therefore lacking a true negative control (Coady *et al.,* 2010). Both inhibitors of the TH production (PTU and SP) resulted in goiter formation (enlarged thyroid). Extreme hyperplasia and hypertrophy of the thyroid cells were consistent with most reports (Grimm *et al.* 2009; Opitz *et al.,* 2005; 2006; 2009; Tietge *et al.* 2010). But, the histological appearance after a 21-day exposure to the two compounds was quite different. Sodium perchlorate resulting in extreme follicular collapse and disappearance of colloid. PTU on the other hand show presence of colloid but very much depleted. Opitz *et al.,* (2009) and Tietge *et al.* 2005; 2010) reported on difference found when using different goiterogens and suggested that when selecting negative controls, especially when conducting gene expression studies, one must be mindful of the mechanism of action and difference in histopathology. Thyroxine as a positive control significantly accelerated development, displaying atrophy and small amounts of stored colloid (Grim *et al.,* 2009; Miyata and Ose, 2012). One concern when testing unknown chemicals is whether subtle changes in the histology of the glandular tissue will allow for correctly recognition of mild antagonism or stimulation.

Field studies

Water collected from the Kuils River- Eerste River represents the first study to use the XEMA set-up with water collected on a regular basis for exposure of premetamorphic tadpoles (NF-stage 51) in a semi static XEMA set-up for 21 days. Tadpoles exposed to water from five different sites in the catchment mostly (four out of the five sites) showed acceleration of development. The latter observation is in contrast to the increase in time to metamorphosis recorded in Rana pipiens tadpoles exposed to WWTP effluent (50% diluted and 100% samples) (Sowers *et al.,* 2009). In the Sowers *et al.,* (2009) very little difference in thyroid histology was reported, at most, it seems that the differences in hyperplasia may be attributed to differences in developmental stages. However, tadpoles exposed to water from one site (downstream of Zandvliet discharge) showed a significant deceleration in development compared to the other sites, but not significantly different from the laboratory control tadpoles. Although the catchment was close-by it still was challenging to get fresh exposure water collected and transported to exposure laboratory on a daily basis. However, mortality of tadpoles was low and selected water samples were used undiluted after initial pre-exposure testing for lethal toxicity. It is well-recognized that industrial and waste water

treatment plants, discharging into catchments may contribute several compounds that have the potential to disrupt the endocrine system of aquatic wildlife as well as humans exposed to these water sources (Daso *et al.,* 2012; Olujimi *et al.,* 2012).

Waste Water Treatment Plants

The **City of Windhoek** study was included to evaluate the practicality around conducting a long distance study (using a supply-train of water for a full 21-day XEMA exposure). From this study, we learned that it is indeed possible but that the commitment to a continued water supply must be ensured.

Because of the limited samples that could be transported, two of the four sites were selected to represent samples of final treated water (BR & GAC) and two as representing samples of inflow sources (VB & RM). Apart for the samples obtained after the activated charcoal filter, other sites did not show any significant effects on tadpole development. In the Zandvliet study (this report) no thyroid disruption was found in the final effluent, despite reports of phthalates and phenols detected (by analytical chemistry) by Olujimi *et al.,* (2012). Along a similar line, Jugan *et al.,* (2009) using an *in vitro* cell-based assay showed that potential thyroid disruption was only found in Paris WWTP influents that they studied. Ishihara *et al.,* (2009) also showed that although the inflow into a STP (in Thailand) may have thyroid inhibition activity (based on three *in vitro* assays) the treated effluents did not show strong inhibitory effects and could the conclude that wildlife will not suffer thyroid disruption from treated discharges. Evidently, more research is needed to confirm the removal of thyroid disruptors from treated wastewater in general. It is difficult to explain the high mortality and some evidence of thyroid inhibition in the post-GAC sample (over a period of 21 days and including six sampling events over three weeks with replicate groups included). The GAC filtering was renewed prior to the sampling and the possibility exists that some unknown compound (s) may have contaminated the GAC system. The histopathology confirmed the developmental deceleration and indicated histopathology that points to goiterous activity.

The primary aim of the City of Cape Town (Zandvliet WWTP) study was to set-up and evaluate a simple flow-through system to screen final effluent of a large WWTP for potential thyroid disruption using developing *Xenopus laevis* tadpoles to study differential development after an exposure period of 21 days. Although the XEMA set-up has been developed to standardize the testing of man-made chemicals for thyroid disruption (OECD, 2007a;c) it has been used to screen environmental samples as well (Bogi *et al.,* 2003). Initially, the XEMA set-up used a semi-static exposure design but, although technically more difficult to set-up and manage, flow-through systems now largely replace the original semi-

static systems. The advantages in terms of maintaining water quality and chemical concentration at acceptable levels during the exposure period are two of the most important advantages associated with a flow-through system. However, Lutz *et al.,* (2008) has shown that in the case of estrogen exposure, maintaining estrogen concentrations under flow-through conditions may be challenging. Noteworthy was that the use of a flow-through system did not necessarily increase the inter-assay repeatability (Pickford *et al.,* 2003; Lutz *et al.,* 2008). Most studies conducted, including the EPA-OECD validation studies used relatively high flow-rates. Pickford *et al.,* (2003) used a system with a turnover rate of six tanks per day. Lutz *et al.,* (2008) used a flow-rate of 50 L/9-L tank per day resulting in a turnover-rate of seven tank volumes per day (Lutz *et al.,* 2008). Coady *et al.,* (2010) experimented with flow rates and potential effects on the thyroid endpoints. They reported no differences between relative fast rates and much slower rates. They did however show body mass effects when a recycling submersible pump was used. This response likely was a general stress response. The stocking rates in both studies were 40 tadpoles per tank with four replicate tanks included per dilution. The OECD-EPA guidelines recommend the use of 4 L exposure volume, tanks containing 20 tadpoles per tank with a flow of 25 mL/min. In the present study, we used a similar set-up but reduced the flow, 25 L per two replicate tanks per day. The decrease in flow-rate was partly a practical consideration since the water supply and wastewater produced, equate to large volumes. On the other hand if using environmental water, the lower flow rate corresponds to the natural, slow flowing (stagnant) conditions *Xenopus* laevis tadpoles experience in nature. In spite of the low turnover rate in the tanks (three tanks per 24 hours) the tanks remained clean, water quality parameters stayed within the guideline ranges and no mortalities occurred. Lutz *et al.,* (2008) mentioned replacing tanks on a weekly basis with clean tanks or less often but then using siphoning to clean out debris. We also used the siphoning procedure but did not have to replace tanks with clean tanks.

Interestingly, few studies report on the volume of wastewater produced by a relatively fast flowing flow-through system and how this is treated and handled. Testing chemicals in a flow-through system will require a decontamination facility before recycling the wastewater.

Based on the initial results of a first XEMA study (Fourie, 2005) on water collected from the Kuils-Eerste River system the aim of this study was to confirm the indications of thyroid disruption downstream of the Zandvliet WWTP. In addition, Olujimi *et al.,* (2012) reported the Zandvliet WWTP to be the most polluted since it receives most of its wastewater from an adjacent informal settlement. This study reported relative (to other WWTPs) high DBP concentrations upstream of the WWTP, but most notably showing high DBP concentrations in the final effluent. The sampling for the Olujimi *et al.,* (2012) study

was conducted in the 2010/2011 seasons. The present study was conducted in the summer of 2010 (corresponding to Q1 in Table 7 of the Olujimi *et al.,* 2012 study). This is the season when the highest DBP concentrations were reported. A study by Shen *et al.,* (2011) showed that *Xenopus laevis* tadpole development were delayed at 10 and 15 mg/L DBP concentrations and that disruption in the expression of thyroid hormone response genes may occur at even lower concentrations (2 mg/L). Although the influent DBP concentrations at Zandvliet WWTP ranged from 16-36 mg/L the final effluent concentrations were lower than 10 mg/L (3.5-7 mg/L). In the present study, no significant developmental delay occurred after the 21-day exposure using the flow-through setup. The removal of thyroid disruption activity during the treatment process in a WWTP was confirmed by Jugan *et al.,* (2009) only showing potential disruption in the influent samples. Similarly, Li *et al.,* (2010) showed thyroid disruption in the influent of a Chinese WWTP but reported an 80% removal of anti-thyroid activity in the effluent. Sowers *et al.,* (2009) reported an increased time to metamorphosis (suggesting inhibition of thyroid activity) in 50% and 100% WWTP effluent in tadpoles of Rana pipiens. However, the histology of the thyroids did not show clearly that the thyroid was inhibited, showing hypertrophy or hyperplasia in the 100% effluent exposure. In the resent study no developmental difference were recorded and hardly any difference in histopathology noted.

In conclusion, it is evident from the literature that more studies are needed on WWTP effluents, in particular *in vivo* exposures, but conducted along with *in vitro* bioassays and analytical chemical analyses. A local analytical chemistry study (Olujimi *et al.,* 2012) confirmed the presence of potential thyroid disruptors like phthalates in the influent and also in the effluent (although at lower concentration) of several WWTP in and around the City of Cape Town. In the present study we investigated the thyroid disrupting effects of WWTPs (including the reported most toxic one from the Olujimi *et al.,* 2012 study) and samples from the Kuils River – Eerste River catchment with several WWTP discharges and could not confirm thyroid disruption. Similarly, the effluent of a reclamation plant where water is blended with treated water as drinking water (City of Windhoek), could we not show clear evidence of thyroid disruption. A post-activated charcoal filter sample at this plant did however show evidence of mild thyroid disruption, but since tadpole mortality was also high in this sample, it may have been the result of a temporary toxic effect associated with the replacement of the activated charcoal. It is clear that, in order to use analytical data towards individual health and/or ecological risk assessment models; there is an urgent need for multidisciplinary research linking analytical chemists and biologists to understand the real risk of ED in the aquatic environment (Kloas and Lutz, 2006). From the biological perspective, it seems that although several *in vitro* bioassays may address certain aspects of

thyroid disruption, the exposure of developing *Xenopus laevis* tadpoles in the XEMA approach represent a valuable tool to assess the potential for thyroid disruption and that a flow-through system could be valuable in assessing environmental samples. From the current literature, it is also evident that more attention should be given to thyroid disruption potential of effluents from WWTPs before making any conclusions.

The *Xenopus* Metamorphosis Assay (XEMA) or Amphibian Metamorphosis Assay (AMA) is a 21-day assay for thyroid disruption included as a Tier 1 screening assay for potential thyroid disruption in the Endocrine Disruptor Screening Programs of the USA Environmental Protection Agency (USA-EPA) (US-EPA, 2009) and the Organization for Economic Co-operation and Development (OECD) (OECD, 2009). This assay has undergone extensive validation and finally reviewed by an independent Review Committee convened by the USA-EPA. Most countries associated with OECD and the USA-EPA endorsed and is using XEMA as a Tier 1 screen. However, in developing countries the XEMA set-up as suggested in the XEMA SOP, in particular the flow-through system, may not be possible or practical. The aim of this study was to conduct a controlled XEMA along the lines of the XEMA SOP and compare the outcome to the validation results obtained in the inter-laboratory trails (OECD, 2007c). The main laboratory experiment was set-up as a semi-static exposure study and a simple flow-through exposure system for environmental water was tested. In contrast to the initial validation, studies the present study did not exposed pre-metamorphic tadpoles to a series of concentrations but rather selected an optimal concentration for the particular chemical from the previous validation studies. The outcome of the laboratory exposure study produced similar results with the control chemicals as what was achieved in the validation studies. During the review, the panel had several criticisms regarding the use of an *in vivo* study stretching over a period of 21 days as a Tier 1 test. But, because no reliable or validated *in vitro* bioassay was available, it was decided to continue to recommend XEMA as Tier 1 screen. There are also criticisms that XEMA/AMA may not be a reliable assay for always detecting HPT-active chemicals (Coady *et al.,* 2010). The concern is that XEMA does not have a true negative control. Another concern mentioned, is that as a Tier 1 assay it may lack specificity to detect HPT axis EDCs because XEMA/AMA includes apical endpoints, which may be altered by generalized stressors (Denver, 1997). It is well known from studies by the Denver group that environmental stress may accelerate metamorphosis (Denver, 1997; Boorse and Denver, 2002; 2003; Denver, 2009)

Conduction XEMA in a laboratory set-up, within the guidelines proposed by USA-EPA and OECD is quite a labour intensive operation. It is started with successful breeding tadpoles and rearing healthy tadpoles to NF stage 51. During the exposure period of 21

days, intensive care of the tadpoles is needed and feeding and water quality monitoring conducted on a daily basis. In a semi static situation water change happens three times a week. Using a flow-through setup will require large volumes of water and produce large volumes of effluent. Very few studies mention the removal of chemical effluents.

The main trait used as key endpoint in XEMA is the NF developmental stage when using *Xenopus laevis* or Gosner staging (Gosner, 1960) when using other anuran species. Staging may also require knowledge and experience with tadpoles and could be a point of error. Measurement of hindlimbs and forelimbs require a dissection microscope with a camera lucida and image analysis software. Most of the studies using XEMA to answer questions regarding potential thyroid disruption of a particular chemical confirmed that the histopathology study associated with the XEMA approach, besides the NF developmental stage, may be another key element of the set-up (OECD 2007a; Grim *et al.,* 2009). Histological sectioning of the thyroids represents another labour intensive procedure. Moreover, interpretation of histopathological conditions requires extensive experience and training. One of the criticisms regarding the histological studies is the selection of post-exposed individuals for histopathology. Mostly at the conclusion of the 21-day exposure period tadpoles of different NF developmental stages will be represented in the group. Because the thyroid grow and develop as the tadpole grow and develop, the stage of development should be taken into consideration. Ideally, tadpoles of similar NF stage (normally the median stage exhibited in the Control group) should be included to make comparison meaningful. Therefore, all individuals should first be staged before selecting individuals for histopathology (Miyata and Ose, 2012). In cases (as is normally seen in the inhibitory and stimulatory controls) where extreme developmental deviations from the control are experienced, the histological comparison may be a problem and the histopathology difficult to compare. In addition the fact that even in the control minimal hyperplasia may be present, since the thyroid of tadpoles in the pro-metamorphic stages are still increasing as tadpoles grow to metamorphosis. Histopathology reading and analysis may prove difficult to the non-specialist and will require training and experience.

Keeping in mind that XEMA is used as a Tier 1 screening tool, it holds promise to be used with environmental water samples. In the present study, several opportunities were used to conduct XEMA using environmental samples. Distance of travel and volume of samples include some of the practical problems. The Namibian samples (100 L per collection) were transported by overnight road transport over a distance of 1500 km three times a week for three weeks (900 L in total). In a study concerning the potential thyroid disruption on a Swaziland cattle farm (unpublished data), smaller samples were collected (5 L samples per site over two weeks). Although the seven day sampling did not prove

valuable with a normal XEMA, relying heavily on NF staging, recently it was suggested that detailed histological and gene expression studies allow for assessment after a short exposure, for example, eight days (Tietge *et al.,* 2010). In the Swaziland example, a 14 exposure with a smaller sample size was used and differential growth and stage development was noted, however, the study used pre-metamorphic tadpoles as showed in the study of Tietge *et al.,* (2010) pro-metamorphic tadpoles may be a better choice for short-term exposures. In the case of the Zandvliet WWTP study, a simple flow-through set-up using an eighteen channel peristaltic pump was used. In order to overcome the challenge of transporting large volume of water, the flow rate was reduced drastically. Exposed tadpoles all survived and growth and development was normal. Coady *et al.,* (2010) investigated the effect of flow rates on developing tadpoles, specifically because high flow rates may stress tadpoles not experiencing high flow rates in nature, like for example *Xenopus* laevis (Du Preez and Carruthers, 2009) and reported body mass effects in extreme fast flow conditions (3 L/min). In the Zandvliet study, the value of *in vivo* exposure was also shown, with analytical chemistry suggesting relative high concentrations of phthalates and phenols in the final effluent (Olujimi *et al.,* 2012). These compounds are known to inhibit the thyroid significantly (Shen *et al.,* 2011). However, in the present study, after a 21-day exposure no developmental effects were found although the histopathology analysis may suggest some enlargement (hypertrophy) of the thyroids with accompanying hyperplasia evident. Similarly, samples collected in the Kuils River – Eerste River catchment where several WWTPs discharge treated effluent, no extreme developmental deceleration were found. Rather, some suggestion of acceleration of development was recorded. These results may suggest that the complex mixtures containing a variety of anthropogenic compounds (many with EDC activity), flowing in these seemingly polluted rivers need more than analytical chemistry to show EDC activity. The XEMA Tier 1 screen is well suited to test for thyroid disruption.

4 CHAPTER 4: SHORT-TERM *IN VIVO* EXPOSURE: CHANGE IN GENE EXPRESSION PROFILES

Prepared by ME Esterhuyse

4.1 *Xenopus* laevis as model

4.1.1 Introduction

Developmental disruptive effects of environmental chemicals during early development of amphibians can be studied using incidence of morphological malformations (Bantle, 1994; Bantle *et al.,* 1998) and/or temporal and histological effects during metamorphosis (XEMA). However, these approaches on their own may overlook disruption at the genetic level (Veldhoen and Helbing, 2001; Veldhoen *et al.,* 2002). Toxicogenomics as an approach to understand the mechanisms underlining the action/disruption of environmental chemicals acting as endocrine disruptors, has grown exponentially (Wintz *et al.,* 2006; Lema *et al.,* 2008; Li *et al.,* 2011), mainly because this approach allow for short-term exposures of fish at an early developmental stage when expression of several genes controlled by thyroid hormone (TH) could be used as biomarkers. One of the advantages of using a quantitative gene expression approach, is that short-term exposure experiments (24-48 hours) can be conducted as opposed to a XEMA *in vivo* exposure approach that will take 14-21 days to complete. Thyroid hormone (T4 and/or T3) regulates several events during amphibian metamorphosis mediating its action through binding to nuclear thyroid receptors (TRs), acting as ligand-dependent transcription factors. In vertebrates, two genes are known to encode TRs, namely TRα and TRβ (Mangelsdorf *et al.,* 1995; Turque *et al.,* 2005). It is known that TRs are expressed at low levels in the premetamorhic phase of development but subsequently the potential upregulation of TRs by T4/T3 exist. It has been reported that TH may affect the expression of 45 genes in the tail, 34 in the brain and no less than 120 in the limbs within the first 48 hrs of exposure (Brown *et al.,* 1996; Denver *et al.,* 1997). Veldhoen *et al.* (2002) identified 79 genes whose mRNA levels were altered in the tails of *Xenopus* tadpoles during metamorphosis and the expression of 26 genes were altered following the exposure to the herbicide, acetochlor (Crump *et al.,* 2002). Helbing *et al.,* (2003) developed a cDNA array including an extensive novel frog cDNA library (420 genes) and identified 93 TH-responsive genes. They have shown that during the premetamorphic phase (within NF stage range, 52-54), because the thyroid is not active few of these genes express but have the potential to be unregulated following TH stimulation (from exogenous sources) (Tata, 1994; Shi, 1999). Since the induction of metamorphosis is dependent on the expression and upregulation of the TH-responsive nuclear receptors, TRα and TRβ these have been the

focus of most studies. Although TRα expression increase early during the developmental programme (Shi, 1999) and is a critical component of the TH response, TRβ received most of the attention since it is thought that TRβ is associate directly with the genetic programme associated with metamorphosis (Helbing *et al.,* 2007a). When TH (in particular, T3, originated peripherally by transformation of thyroxin by diodinase enzymes) binds to and activates the TRα and TRβ, these nuclear receptors functions as heterodimers with 9-cis retinoic acid receptor (RXR) to modulate gene transcription (Ranjan *et al.,* 1994; Heimeier and Shi, 2010a). The fact that expression of TRs can be induced by external THs or mimics, makes it an excellent developmental period to study the effects of exogenous TH or mimics on the expression of TRs (Kloas, 2002; Opitz *et al.,* 2006b; Veldhoen *et al.,* 2006). One of the most sensitive genes induced by TH is TRβ (Denver *et al.,* 1997; Crump *et al.,* 2002), specifically responsive to T3 (Jagnytsch *et al.,* 2006). Therefore, although there may be many genes that are responsive to TH stimulation, TRβ remains one of these with potential to be used as molecular biomarker to study EDC effects on TH action in *Xenopus laevis* (Opitz and Kloas, 2010).

The aim of this section was to exposure to *Xenopus laevis* tadpoles to TH (T3) and Bisphenol A for 48 hrs and then quantify the expression of TRβ to evaluated the potential of this suggested sensitive bioassay to detect thyroid disruption by EDCs following a short-term exposure. I this study published methods (Zhang *et al.,* 2006; Helbing *et al.,* 2007a) using *Xenopus laevis* tadpoles at NF-stage 52 (Late premetamorphic, prior to endogenous elevated T3 levels) to illustrate the appropriateness of this assay in the South African subcontinent where *X. laevis* is indigenous.

Bisphenol A was selected for this assay since this compound has been implicated as a thyroid disruptor and included in several EDC related studies. It should therefore help to demonstrate the potential of combining morphological and molecular endpoints to show developmental effects associated with TH pathways. In particular, has Bisphenol A also been implicated as having effects on human embryonic and postembryonic development (Heimeier and Shi, 2010). Bisphenol A (BPA) is a monomer of polycarbonate plastics and epoxy resin, a compound that is used in dental composites, baby-feeding bottles and to line canned food containers to name a few. Apart from the adverse effects in the estrogenic steroid biosynthesis pathway (Lutz and Kloas, 1999), BPA has recently been reported to suppress organogenesis resulting, in amongst other, malformation of the head region and scoliosis in *Xenopus laevis* and in higher concentrations, it may block tri-iodothyronine-inducible resorption of tail segments in larvae before metamorphosis of the same species (Iwamuro *et al.,* 2003), however, Heimeier & Shi (2010) suggested several possibilities of BPA interacting with the binding of TH with TR (Zoeller, 2005). Several lines of evidence

suggest that BPA can bind to and antagonize T3 activation of TR (Heimeier and Shi, 2010). Interestingly, Heimeier *et al.* (2009) reported that BPA on its own had little effect on transcription, but when in the presence of T3, had and antagonizing effect on the development of *Xenopus laevis*. It was therefore suggested that BPA might function as an inhibitor. Similar results were reported using fluorescent transgenic *Xenopus* laevis tadpoles (Fini *et al.,* 2007).

4.1.2 Material and Methods

4.1.2.1 Xenopus laevis tadpoles

All *Xenopus laevis* premetamorphic tadpoles used in this experiment were bred and reared in house from breeding stock of the Ecophysiology laboratory, Department of Botany and Zoology, Stellenbosch University. See above for details of breeding, rearing and maintenance. All aspects of this study were conducted in compliance with the guidelines of OECD, and locally, SANS and received ethical clearance from the Stellenbosch University Animal Ethics Committee.

4.1.2.2 Exposure study

Premetamorphic tadpoles, NF- Stage 51-52 (Nieuwkoop & Faber 1956), were transferred to an experimental facility where they were distributed randomly, six tadpoles per two litre glass jar, and left to acclimated (temperature 22 ± 1℃) in aerated exposure water RO water with salt added, 2.5 g\10 L)) for a day (24 hrs). During the experiment, tadpoles were exposed to chemicals as follows: (A) 10 nM T3 (3,3', 5-Triiodo-L-thyronine, Sigma, Germany)[NOTE: Crump *et al.* 2002 used 100 nM T3 as positive control but for this study we used 10 nM T3 in accordance with Opitz *et al.* (2006b)], (B) 25 µM BPA (Bisphenol A Aldrich, Germany), (C) 10 nM T3 and 25 µM BPA, (D) a vehicle treatment containing DMSO served as control. Each exposure group was duplicated (2 x 6 tadpoles per group). Chemical applications were not renewed and tadpoles were not fed during the during the test period (48 hrs).

Tadpoles were euthanized using 0.01% Benzocaine (Haynes Mathews, Ltd., South Africa) and preserved in RNAlater (Ambion Inc., Austin, TX, USA) at 4°C.

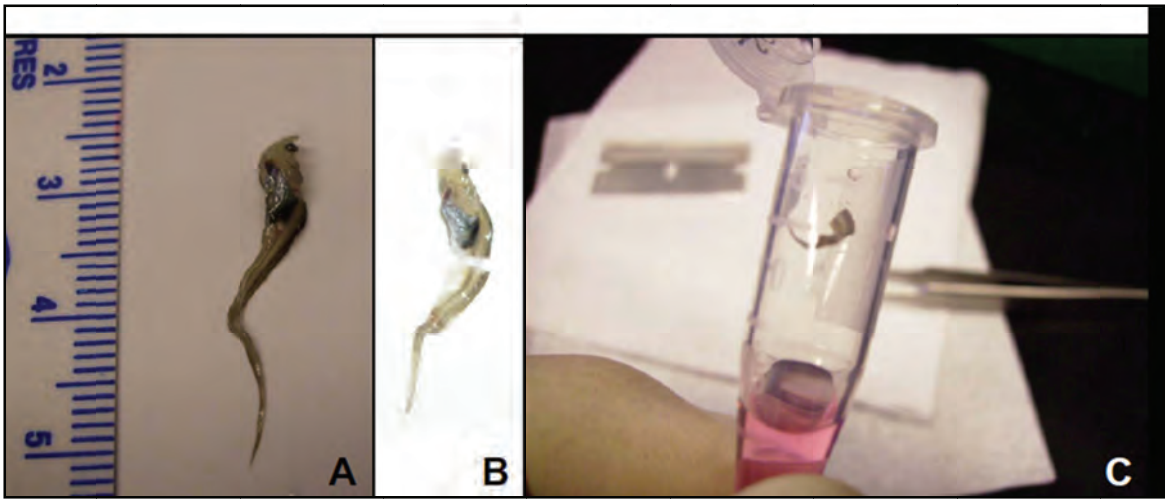

Figure 42. *Xenopus laevis* tadpoles (N&F stage 52) prepared for mRNA isolation: A: a sample taken from storage in RNAlater, rinsed and dabbed with a tissue paper wipe, B: section anterior of vent to obtain tail tissue, C: tail tissue sample going into Trizol reagent.

4.1.2.3 Total RNA isolation and cDNA preparation

Detail procedures were according to Crump *et al.* (2002b). In brief: total RNA was prepared from dissected tail tissue of individual samples using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA pellets were washed with 70 % ethanol and redissolved in 30 to 60 µl of DEPC-treated water. RNA yields were quantified spectrophotometrically with a Nanodrop® (ND 100) system (NanoDrop Technologies, Inc. USA) and stored at -70°C. First strand cDNA was prepared from 2 µg of total RNA using oligo d(T)15 primers and SuperScript III RNase H- MMLV reverse transcriptase (Invitrogen, USA) as described by the manufacturer. Samples were diluted 20-fold prior to gene expression determination and stored at -20 °C.

4.1.2.4 Quantitative gene expression analysis

Detail protocols were according to Crump *et al.* (2002). In brief: primers for analysis of gene expression by quantitative PCR (QPCR) were obtained from Viagenex (Canada). Gene expression was quantified using a Stratagene MX4000 real-time quantitative PCR system (Stratagene, USA). Each 15 µl QPCR reaction contained 2 µl of first-strand cDNA, 1x buffer (Viagenex, Canada), 0.2 mM dNTPs, 2.67 mM $MgCl_2$, 0.27 µM of each primer and 1 unit of Platinum Taq polymerase (Invitrogen, USA). The thermo-cycle program included 95ºC (9 min), followed by 40 cycles of 95ºC (15 sec), 55ºC for (30 sec) and 72ºC (45 sec). Each QPCR run included control reactions containing no cDNA template and a standard concentration of each target DNA amplicon (4 x 104 copies/reaction). Ct values obtained across independent amplification runs for a given gene target were used to transform the

data into gene levels expressed as copy number per µl. Triplicate reactions were performed for each sample. Corrections for possible discrepancies of amount cDNA input in samples were made by one housekeeping gene, the internal invariant Ribosomal protein L8.

4.1.2.5 Statistical analyses

We made use of basic statistical models to handle parametric and non-parametric data within the software package STATISTICA© v7 (StatSoft Inc., USA). Data was tested for normality and equal variance among groups. For parametric data sets, we used Analysis of Variation (ANOVA) to test for significant variation ($P<0.05$) together with Holm-Sidak's Multiple Comparison test to test for significance among groups. In cases of non-parametric data sets, Kruskal-Wallis Analysis of Variance (ANOVA) on ranks followed by Dunn's Multiple Comparison Procedure was used to test for significance among groups. Quantitative values were always tested both as normalized data or as the log10 of these values to see if significance increases with transformation.

4.1.3 Results

4.1.3.1 Quantitative TRβ transcript levels in T3 induced samples

Not all data from our data set was normally distributed according to Shapiro-Wilk's W test and we therefore, we used Kruskal-Wallis ANOVA to compare exposed groups with each other (Table 9). Thyroid hormone (T3) significantly ($p < 0.05$) induced TRβ transcription after a 48 h exposure (Figure 43). Bisphenol A did not alter expression of TRβ in tail tissue significantly (Table 9), but in combination with T3, BPA increased the level of TRβ transcript levels significantly (Figure 43 & Table 9). However, the combined induction was not significantly more than that of T3 alone (Figure 43 & Table 9).

Table 9. Krustal-Wallis Test done on normalized data for TRβ transcription levels as has been measured from tail tissue in *Xenopus laevis* NF-stage 52 (Nieuwkoop & Faber, 1956) tadpoles. Bold figures indicate significant difference at p < 0.05.

	Control	T3	BPA	T3 + BPA
Control		3.627562	0.589775	4.687534
T3	3.627562		3.186058	1.185610
BPA	0.589775	3.186058		4.301636
T3 + BPA	4.687534	1.185610	4.301636	

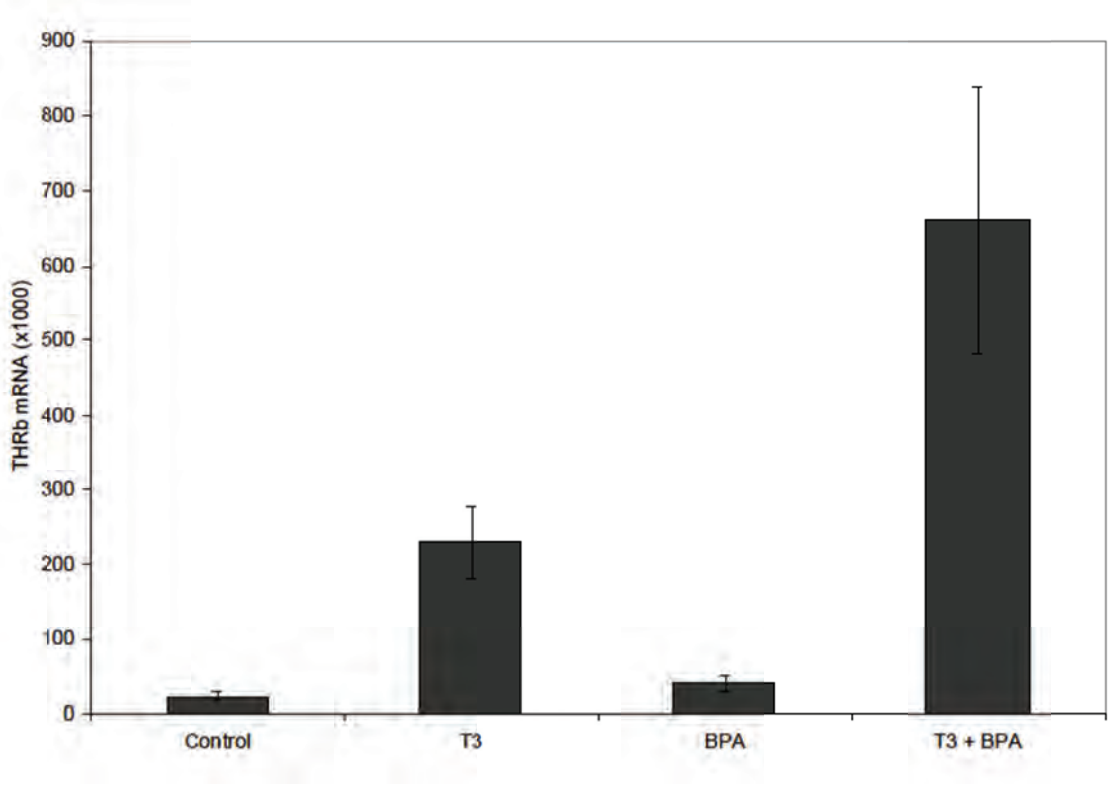

Figure 43. Relative expression levels of TRβ mRNA in *Xenopus laevis* tadpole tail tissue 48 hr post-treatment as determined by polymerase chain reaction. The treatments included are vehicle control, 10 nM T3, 25 µM Bisphenol A, and a combination of 10 nM T3 and 25µM Bisphenol A. Each group included 12 tadpoles. Data are presented as normalized number of copies.

4.1.4 Discussion

The aim of this exposure experiment was to illustrate the potential utility of TRβ mRNA as a molecular biomarker to study disruption of TH action using *Xenopus laevis* as model. The goal was accomplished by showing that 10 nM T3 did significantly up-regulate the expression of TRβ mRNA and that potential thyroid modulators could be screened in a similar way. The present results corroborate the results of several studies that optimized the testing conditions and *Xenopus* laevis bioassay (Crump *et al.,* 2002; Veldhoen *et al.,* 2002; Opitz *et al.,* 2006b; Opitz *et al.,* 2006c; Zhang *et al.,* 2006). The study also confirm the statement made by Opitz *et al.* (2006b) that "one of the greatest advantages of the *Xenopus laevis* metamorphosis model is the stereotypic manner in which premetamorphic tadpoles respond to treatment with biological active TH agonists". In the present study we confirm the repeatability/reproducibility of the induction of TRβ mRNA as a sensitive TH-responsive gene (Crump *et al.,* 2002; Opitz *et al.,* 2006b), underline the potential for its use as a short-

term *in vivo* testing approach to screen for potential thyroid modulation by EDCs (environmental chemicals). Opitz *et al.* (2006b) highlighted several aspects, including that TRβ RNA is expressed ubiquitously in tadpole tissue, and that the level of expression can precociously be upregulated by exogenous TH or environmental chemical agonists. The present study therefore supports the view that TRβ mRNA expression following short-term (12-48 hr) exposure of premetamorphic tadpoles to potential EDCs may be an attractive and simple bioassay to assess environmental compounds for TH-interaction. It is clear from the recent literature that availability of primers and detailed/standardized protocols (Crump *et al.,* 2002; Veldhoen *et al.,* 2002; Helbing *et al.,* 2003; Opitz *et al.,* 2006b; Opitz and Kloas, 2010) allow for a much wider application.

Bisphenol A (BPA) was included to show how a known environmental contaminant could be screened for potential disruption of the TRβ mRNA program. In the present study, BPA did not significantly upregulated the expression of TRβ mRNA during the 48 hr exposure. However, the combination of T3 and BPA significantly increased TRβ transcript levels ($P<0.05$) compared to the Control and BPA treatment alone. Very much a similar results came from a study with a co-exposure of the herbicide, acetochlor in combination with T3 (Crump *et al.,* 2002) demonstrating that not only can the detection of antagonistic effects of EDCs be evaluated but also agonistic (potentiating or synergistic) effects of EDCs be demonstrated. Turque *et al.,* (2005) and Fini *et al.,* (2007) using transgenic *Xenopus laevis* (expressing a TH fluorescence gene), pre-treated premetamorphic tadpoles with the T3 before exposing these to either the herbicide, acetochlor alone or in combination with T3. The pretreatment allowed for a more rapid detection of a TH agonist like the herbicide acetochlor. In the present study, similar to the Crump *et al.,* (2002) and Turque *et al.* (2005) studies, BPA on its own did not upregulate TRβ mRNA but in combination with T3 it did. Although BPA did not affect the expression of TRβ m RNA, it did in the presence of T3 amplify the expression of TRβ mRNA, therefore potentially modified the physiological effects of TH. Several studies have shown that BPA can bind to, and antagonize T3 activation of TRs (Heimeier *et al.,* 2009; Heimeier *et al.,* 2010). Heimeier *et al.,* (2010) suggested that BPA inhibited the transcriptional activation of the TRβ promoter by T3 but had little effect on the promoter on its own. Heimeier *et al.,* (2009) did indeed report the down-regulation of several TH-responsive genes when exposing tadpoles to T3 and BPA, however they did not find a similar effect when considering TRβ. Iwamuro *et al.,* (2003; 2006) on the other hand reported the down-regulation of TRβ when BPA was combined with a T3 exposure. The Heimeier *et al.,* (2009) study, and the present study, could not confirm this down-regulation of TRβ when in the presence of T3. However, comparing the TRβ expression responses in the two duplicate exposures we found some variation, but still could not show a down-

regulation of TRβ mRNA. Clearly, more research is needed to understand and confirm the potential down regulation of the TRβ mRNA expression when T3 is in the presence of BPA.

In conclusion, this study showed that using the expression of TRβ mRNA as sensitive biomarker in a short-term bioassay using premetamorphic *X. laevis* tadpoles hold great potential to screen for thyroid disruption before conducting the labour intensive longer-term *in vivo* XEMA screen.

4.2 Mossambique tilapia (Oreochromis mossambicus) as model

4.2.1 Introduction

Environmentally relevant chemicals, including polychlorinated hydrocarbons, polycyclic aromatic hydrocarbons, organochlorine pesticides, chlorinated paraffins, organophosphorous pesticides, carbamate pesticides, cyanide compounds, methyl bromide, phenols, ammonia, metals, acid loads, sex steroids, and pharmaceuticals are known to have acute or chronic effects on the thyroid cascade in the more than 40 teleosts tested to date. In a review by Brown *et al.,* (2004) the authors describe thyroid endpoints to serve as biomarkers to exposure to environmental pollutants in addition to the endpoints well established in the steroidogenic pathway. Mechanisms underlying thyroid changes and their physiological consequences have become more and more known/understood in the past decade (Brown *et al.,* 2004; Zoeller and Tan, 2007; Sorensen *et al.,* 2008; Zoeller, 2010; Zoeller *et al.,* 2012). Moreover, this line of research is particularly complicated by the fact that the thyroid cascade may respond indirectly and it has considerable capacity to compensate for abuses that otherwise would disrupt thyroid hormone homeostasis (Brown *et al.,* 2004).

The development of bioassays that measure changes in gene expression show great potential in the detection of thyroid active agents in water (Power *et al.,* 2001; Zhang *et al.,* 2006). In this regard, changes in gene expression profiles associated with amphibian metamorphosis, in particular, *Xenopus laevis*, have been well-studied and used to screen for chemical interaction with the thyroid endocrine system (Zhang *et al.,* 2006; Connors *et al.,* 2010; Opitz and Kloas, 2010). The initial goal was to use the role of the thyroid endocrine system in metamorphosis to identify environmental chemicals or activity in environmental waters that may alter hormone-regulated gene expression programs. The use of molecular endpoints is recognized and changes in gene expression, specifically, TH-dependent gene expression, can be detected by 48 h following exposure to exogenous TH (Helbing *et al.,* 2003). During amphibian metamorphosis morphological change requires hormone-dependent initiation of tissue-specific gene expression and it has been shown that the

expression of TH-receptors (specifically, TRβ) show a strong relationship with TH exposure. Increased TRβ mRNA levels suggesting TH-like (agnostic) activity (Zhang *et al.,* 2006). It is also possible to assess the competitive or synergistic effects of EDCs on T3 or T4 TH interaction with thyroid hormone receptors. Although short-term gene expression end-points used with the highly sensitive QPCR methodology show great promise as an effective screening tool, very little is known about the expression of TH receptors during development in fish and little is known about the effect of environmental chemicals on the specific thyroid dependent gene expression, specifically, thyroid hormone receptor mRNA associated with increased levels of TRs. Similar to tadpole studies, small fish species or juvenile fish could be used to study relative TR mRNA gene expression in short term exposure assays (either with collected water, or extract of organics or to study specific chemicals, individually or in mixture).

Thyroid responsive genes are being regulated via thyroid hormone receptor (TR) mediation – the latter being DNA-binding transcription factors that function as molecular switches in response to specific ligands (Wu and Koenig, 2000; Wu *et al.,* 2001). TRs, along with receptors for steroid hormones, retinoids and vitamin D are part of the nuclear receptor superfamily (Mangelsdorf *et al.,* 1995; Esterhuyse, 2008). TRα and TRβ, two distinctive genes, code for various TRs, including TRα, TRβ1 and TRβ2 (the latter two known to be translated especially in mammals (Williams, 2000) and *Xenopus laevis*, whereas to our knowledge teleosts only produce one homologue of TRβ) (Wu and Koenig, 2000; Wu *et al.,* 2001). Specific DNA sequences, known as thyroid hormone response elements (TREs), provide a binding site for TRs in order to achieve regulation of thyroid responsive genes. TRs bind to TREs, even in the absence of thyroid hormones. Although TRs are able to bind to TREs as monomers or even homodimers, it more often is reported to bind to multiple TREs as heterodimers with retinoid x receptor (RXR) (Kime, 1998). Mostly arriving from the TRs' ability to bind to TRE's when not ligated, TRs can enhance or inhibit gene expression depending on the nature of theTREs, the hormonal status and the cellular environment (Wu and Koenig, 2000). It is important to consider the role of co-activators within this process as has been described by Kime (1998), Wu & Koenig (2000), Mangelsdorf (1995) since up-or down regulation from basal levels of thyroid responsive genes are largely affected in this manner. The aquatic environment represents the ultimate sink for most anthropogenic contaminants, and it is not surprising that fish are considered one of the primary risk organisms in aquatic ecosystems for EDCs (Kime, 1998). Small fish species have the benefit in that several species can be used as cost-effective models for testing for potential EDCs in exposure systems in the laboratory or in field study situations.

Since freshwater fish are recognized as excellent models to study endocrine disruption in local water bodies, the study on thyroid receptors was extended to the freshwater Mozambique tilapia*, Oreochromis mossambicus*. However, the use of thyroid biomarkers in fish, has not received much attention in the past.

In order to facilitate short-term exposure studies using fish, the objective was to set-up and validate a Mozambique tilapia (*Oreochromus mossambicus*) TRβ mRNA QPCR based assay, very much along the lines of the assay used in *Xenopus laevis* studies (Opitz and Kloas, 2010). Because of this phenomenon where basal levels of thyroid responsive genes can be affected xeno-endocrinologically, studies attempting to report on such disruption need to characterize the transcription levels of the chosen thyroid responsive gene under controlled conditions before attempting any environmental study in this regard. Consequently the current study use the tested and characterized bioassay for determining the transcript levels of TRβ using the South African tilapiine, *Oreochromis mossambicus*, as model – firstly to describe non-induced expression (a) in different tissues, and (b) evaluate differential expression following exposure to control hormone (T3) and selected anthropogenic chemicals, Bisphenol A (BPA) and/or Endosulfan (END), individually and on mixture with T3.

4.2.2 Methods

4.2.2.1 Total RNA isolation and cDNA preparation

Total RNA was prepared from specific tissues of adults or from whole body homogenates of juveniles using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Following resuspension of total RNA in diethyl pyrocarbonate (DEPC)-treated water, samples were treated with DNase I (Promega, USA) for 30 min at 37°C and precipitated with 0.1 volumes 3 M sodium acetate pH 5.6 and 2.5 volumes of 95% ethanol at -20°C. The RNA pellets were washed with 70% ethanol and redissolved in 30 to 60 µl of DEPC-treated water. RNA yields were quantified spectrophotometrically at $Absorbance_{260nm}$ and stored at -70°C. First strand cDNA was prepared from 2 µg of total RNA using oligo $d(T)_{15}$ primers and SuperScript III RNase H^- M-MLV reverse transcriptase (Invitrogen, USA) as described by the manufacturer. Samples were diluted 40-fold prior to gene expression determination and stored at -20°C or used as template for THR cloning as described below.

4.2.2.2 Isolation of *O. mossambicus* TR cDNA

PCR primer sequences for THRa and THRb cDNA in *O. mossambicus* were initially designed from the closely related *Oryzias latipes* cDNA from ovarian tissue of an adult female was used as template to perform long-range PCR for which a reaction consists of 1.5 mM MgCl2, 0.05 mM of each dNTP, 1 µM of each primer (Table 10) and 2.5 Units of SuperTherm Gold Taq polymerase (JMR Holdings, UK) in a 25 µl reaction. To each reaction,

50 ng cDNA was added. PCR reaction volumes were denatured for 9 min at 95°C, after which followed 30 cycles constituting of 30 seconds at 95°C, 30 seconds at 64°C and 3 minutes at 70°C, with a final elongation step of 5 min at 70°C. PCR products were checked for size on a 0.8% agarose gel. Amplified DNA fragments were cloned into pGEM-T Easy vectors (Promega, USA) and transformed into *E. Coli,* DH5α to sequence and confirm the amplicons. Sequences were deposited into NCBI GenBank database.

4.2.2.3 DNA sequencing and sequence comparisons

Plasmid DNA of full-length cDNA was isolated from positive clones detected by colony PCR, and insert DNA sequenced using SP6 and T7 primers on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, USA). The resulting *O. mossambicus* TR sequence information was deposited in GenBank for TRα and TRβ respectively). Sequence analysis of the DNA and derived amino acid sequences was performed using ClustalW software according to (Chenna *et al.,* 2003) and graphic illustrations were prepared with software available in Bioedit Sequence Alignment Editor v7 (Hall, 1999). Sequence alignments were done for various vertebrates by using the BLAST program (Altschul *et al.,* 1997).

4.2.2.4 Gene expression analysis by QPCR

Primers for analysis of gene expression by quantitative real-time PCR (QPCR) were designed for TRα and TRβ from the distinctive genes sequenced (Table 10). Beta (β)-actin (GenBank accession no.AB037865) was used for normalization as has been described before (Esterhuyse *et al.,* 2008a; Esterhuyse *et al.,* 2009) since transcript levels of β-actin did not change. Gene expression was quantified using a using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, CA, USA). Each 15 µl QPCR reaction contained 2 µl of first-strand cDNA (40-fold dilution), 7.5 µl SYBRgreen mix (Sigma, Germany), 0.08 µl reference dye (Sigma, Germany) and 0.27 µM of each primer. The thermocycle program included 95°C (9 min), followed by 40 cycles of 95 °C (15 sec), 31 sec at 65°C and 72°C (45 sec). At the end of each programme a dissociation step was included as confirmation of amplicon size. Each DNA amplification run included control reactions containing no cDNA template and a standard concentration of each target DNA. Triplicate determinations were performed for each sample and the CT values obtained across independent amplification runs for a given gene target which were used to transform the data into gene levels expressed as fold change compared to a standard (a 20 dpf sample without exposure to additional chemicals) according to the $\Delta\Delta$Ct method (Pfaffl, 2001; Bustin, 2002; Rutledge and Cote, 2003; Kubista *et al.,* 2006). A dilution range was generated for each gene target in five-fold dilution increments using the appropriate gene contained in the respective plasmid used for sequencing. QPCR analyses of these were used to determine PCR efficiency (PCR efficiency = 10 (-1/slope) – 1).

Table 10. Thyroid hormone receptors (TRα & TRβ) QPCR in *Oreochromis mossambicus* studies.

Target gene	Application	Primer*	Sequence
TRα	cloning	OLTRa7f	ATG GAA CAC ATG CCC AAG GAG CAG
		OLTRa9r	TCA CAC CTC CTG GTC CTC GAA AAC CTC G
TRβ	cloning	OLTRb7f	ATG ACT GTA TAA AGC TAA AAC
		OLTRb8r	TCA GTC CTC AAA GAC CTC CAG GAA A
	QPCR	OMTHRb1f	GCT TGC AAG TAC GAG GGC A
		OMTHRb2r	AGT CGG TCC CAC GCC GT

** an "f" refers to a sense/forward primer and "r" to the anti-sense/reverse primer*

In each PCR run, standard curves generated using plasmids containing amplicons of interest showed a linear relationship between Ct-values and plasmid concentration with the correlation coefficient (R^2) of 0.997 for TRα. PCR efficiency was calculated as the percentage (%) for the respective genes, whereby the assumptions for the $\Delta\Delta$Ct method (Pfaffl, 2001; Bustin, 2002; Rutledge and Cote, 2003; Kubista *et al.,* 2006) are met. QPCR primer sets as listed in Table 10 were validated for specificity using the authentic gene-containing plasmid. No product was amplified in plasmids with genes other than the specific gene that has been targeted, confirming appropriateness of primer sets.

For the first time, relative quantifications of gene expression of *O. mossambicus* TRβ and TRα was performed. Following short-term exposures (48 hrs), either to specific chemicals or mixtures of chemicals or environmental samples during, tissue samples can be collected in RNAlater reagent (Qiagen, Hilden, Germany) and be stored until RNA extraction. Following QPCR methodology, gene expression data are presented as fold change relative to control animals. In the next deliverable, differential expression of TRβ mRNAs in different tissues and during the developmental programme of *O. mossambicus* will be reported on. In addition, we exposed *O. mossambicus* juveniles to suspected EDCs and quantified TRβ mRNA.

4.2.2.5 Experimental animals

Swim-up fry were obtained from AquaStel (South Africa) and kept in experimental aquaria for two days to acclimatize, and reach age of 20 dpf. Water for the exposure experiment was prepared by using RO (reverse osmosis) water to which is added 250 mg/L iodated sea salt (Cerobos®) was added to provide iodine at 0.01 mg/L in a static exposure regime (see below, Table 11).

4.2.2.6 Chemical Exposure

Juvenile fish (20 dpf) were exposed to test compounds which includes T3, BPA and Endosulfan at concentrations of 10 nM 3,3',5-Triiodo-L-thyronine (Sigma-Aldrich, Steinheim, Germany) ("T3"), 25 µM Bisphenol A (Aldrich, Steinheim, Germany) ("BPA"), 1 µg/L Endosulfan ("Endo1") or 10 µg/L Endosulfan ("Endo10"). Combination exposures included 10 nM T3 with 25 µM BPA ("TB"), 10 nM T3 with 1 µg/L Endosulfan ("TE") and negative control, containing 250 µL vehicle (EtOH, "-C").

Animal husbandry, treatment and handling were done according to the South African Standard: the care and use of animals for scientific purposes (SANS 10386:200X). Juvenile fish, which were used in exposure experiments, were selected at 20 dpf where sex is not morphologically distinguishable but is believed to be already determined (Chang *et al.,* 2005).

4.2.2.7 Total RNA isolation and cDNA preparation.

Anesthetized fish were decapitated, dissected and appropriate tissues/whole bodies were homogenized in TRIzol reagent (Invitrogen, USA) for three seconds, working on ice. Total RNA was prepared from specific adult tissues or from whole body homogenates of juveniles using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Following resuspension of total RNA in diethyl pyrocarbonate (DEPC)-treated water (50 µl), samples were treated with one unit of DNase I (Promega, USA) for 30 min at 37°C and precipitated at -20°C following the addition of 0.1 volumes of 3 M sodium acetate pH 5.6 and 2.5 volumes of 95% ethanol. The RNA pellets were washed with 70% ethanol and redissolved in 30 to 60 µl of DEPC-treated water. RNA yields were quantified spectrophotometrically at Absorbance – 260 nm and stored at -70°C. First strand cDNA was prepared from 2 µg of total RNA using oligo d(T)15 primers (Table 10) and SuperScript III RNase H- MMLV reverse transcriptase (Invitrogen, USA) as described by the manufacturer. Each cDNA sample was diluted 40-fold and stored at -20°C prior to gene expression analysis or used as template for cDNA isolation as described below.

Table 11. Summary of the experimental design for chemical exposure of O. mossambicus to T3, BPA and Endosulfan

Test species	*Oreochromis mossambicus*
Stage	20 dpf
Age criteria	Days post fertilization
Exposure substance	T3, Bisphenol A (BPA), Endosulfan
Exposure component concentrations	T3: 10 nM BPA: 25 µM Endosulfan: 1 µg/L Endosulfan: 10 µg/L
Exposure regime	Static
Pre-exposure	24 h at exposure conditions w/o exposure substance
Endpoints	Trβ mRNA expression
Density	~100 specimens per 10 L
Replication	100 samples per tank, Two tanks per exposure substance
Dilution water/ lab control	Charcoal filtered water containing buffering salt, NaCl (iodated sea salt, 2.5 g containing 0.004 g iodine per 100 g salt)
Test water	Lab control water containing T3, BPA, T3 + BPA, Endosulfan, T3 + Endosulfan.
Exposure vessel	12 L glass aquaria, containing 10 L of test/control water. Aquaria are washed once with Neptune, then rinsed 3x with RO water, then sprayed with Methanol, and finally rinsed 5x with RO water.
Mortality rate in controls	<10%
Feeding	Tilapia pellets, ground finely (AquaNutro, SA)
Light regime	14:10, L:D
Water temperature	27°C ± 1°C
pH	6.5-8.5
Anesthesia	0.5% Benzocaine
RNA isolation	Total RNA, using Trizol and subsequent DNase1 digestion.
cDNA preparation	Promega ImPromII from 2 µg tRNA

4.2.2.8 Quantitative gene expression analysis.

Gene expression was quantified using an Applied Biosystems 7500 (Applied Biosystems, CA, USA) real-time quantitative PCR system. Each 15µl QPCR reaction contained 2 µl of diluted first-strand cDNA, 7.5 µl 2x SYBRgreen mix (Sigma, Germany), 0.08 µl ROX reference dye (Sigma, Germany) and 0.33 µM of each primer. The thermocycle program included 95°C (9 min), followed by 40 cycles of 95 °C (15 sec), 64°C for (30 sec) and 72°C (45 sec). Each sample was evaluated in at least triplicate amplification reactions and each QPCR run included control reactions containing no cDNA template and a standard concentration of each target DNA amplicon (4 x 104 copies/reaction). Amplification DNA products were quality checked using melting curve analysis. Ct-values obtained across independent amplification runs for a given gene target were used to determine relative mRNA abundance by the ΔΔCt method (Pfaffl, 2001; Bustin, 2002; Rutledge and Cote, 2003; Kubista *et al.,* 2006). Beta actin (βactin) was used for the purpose of normalization. The standard curve for quantification using all primer sets was linear over three orders of magnitude with linear correlation (R^2) between Ct and the number of target copies ≥ 0.98 in each case with reactions done in quadruplicate (data not shown). Expression data generated from juvenile fish samples indicating poor RNA quality or suboptimal amplification were not included in further analyses.

4.2.2.9 Statistical analysis

Statistical analyses were performed using the STATISTICA software package v8 (StatSoft Inc., USA). Data was tested for normality and equal variance among groups. For parametric data sets, we used Analysis of Variation (ANOVA) to test for significant variation ($P < 0.05$) together with Holm-Sidak's Multiple Comparison test to determine significance among groups. In cases of non-parametric data sets, we used Kruskal-Wallis Analysis of Variance (ANOVA) followed by a Mann Whitney U test to establish significance between treatment groups.

4.2.3 Results

Characterizing the natural expression of TRβ mRNA (relative to expression in the kidney) showed that expression was generally low in most tissues. Expression of TRβ mRNA varied among tissues (12 tissue types) with the highest expression noted in the gills, liver, muscle and ovarian tissue (gills>liver>muscle>ovary>blood>brain>eye) (data not shown).

Differential expression and the upregulation of TRβ in whole body homogenates following a 48-hour exposure of juvenile fish to T3 controls, BPA and Endosulfan was confirmed (Figures 44 & 45). In both exposure experiments did the T3 treatment result in the upregulation of TRβ mRNA (although only significant in the Endosulfan experiment)

(Figures 44 & 45). The BPA treatment did significantly upregulated the TRβ mRNA expression (Figure 44). The treatment of BPA in mixture with T3 did not show a significant added effect and was the expression not significantly different (P>0.05) from the BPA alone, although significantly different (P<0.05) from the TRβ mRNA expression following T3 treatment (Figure 44). When the experiment was repeated with the pesticide, endosulfan, the upregulation in the T3 control group was significantly different from the clean water control (P<0.05) (Figure 45). Endosulfan treatments (1 µg/L and 10 µg/L) alone did not result in a significant (P>0.05) upregulation or downregulation of TRβ mRNA when compared to the T3 exposure (Figure 45). Endosulfan (10 µg/L) in combination with T3 did show a significant downregulation of TRβ mRNA when compared to the T3 exposure group (P<0.05, Figure 45).

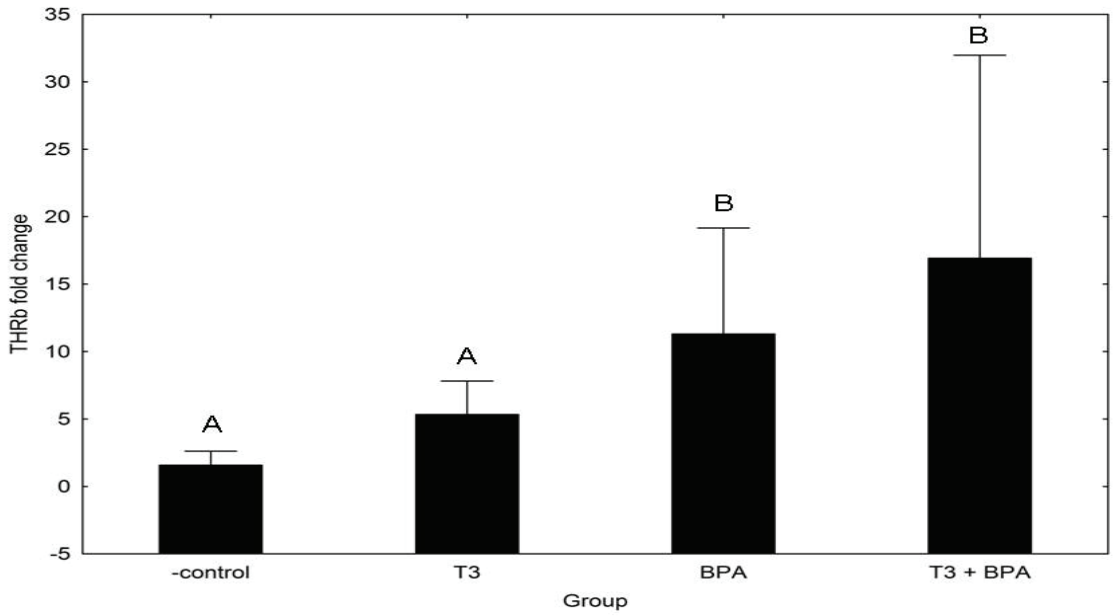

Figure 44. TRβ mRNA expression fold change after a 48 hour exposure of *Oreochromis mossambicus* juveniles (20 dpf) to thyroid hormone (T3), BPA and a mixture of T3 and BPA. Different alphabetic labels above bars indicate statistical significance (P<0.05).

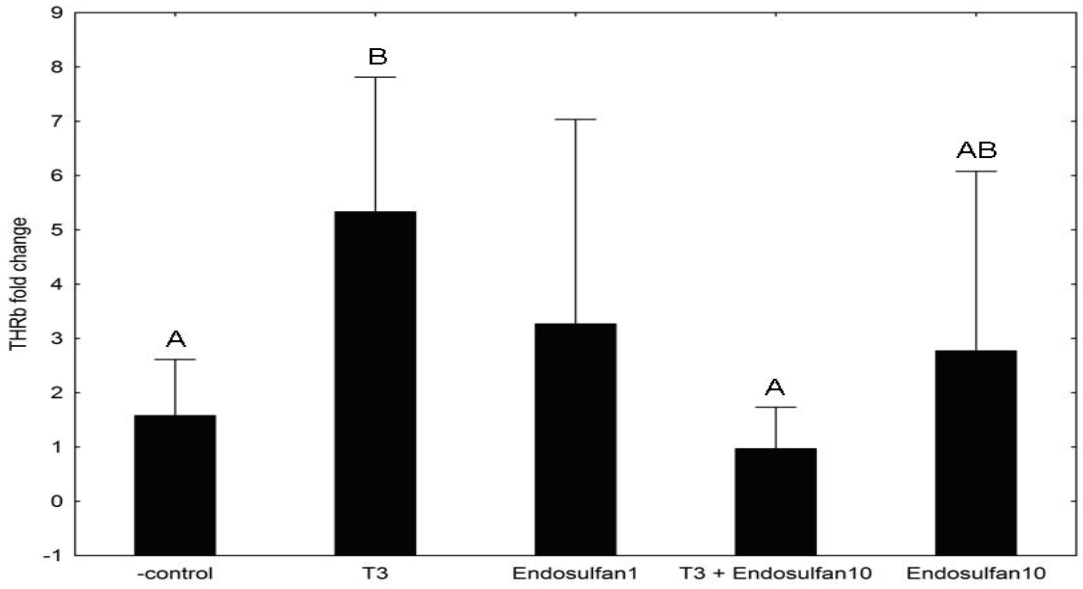

Figure 45. TRβ mRNA expression fold change after a 48 hour exposure of *Oreochromis mossambicus* juveniles (20 dpf) to thyroid hormone (T3), two concentrations of Endosulfan (1 µg/L and 10 µg/L) and a mixture of T3 and Endosulfan (10 µg/L). Different alphabetic labels above bars indicate statistical significance (P<0.05).

4.2.4 Discussion

In this study a qRT-PCR was set up allowing for the isolation of probes corresponding to the TH receptors TRα and TRβ. Tissue expression of TRs in adult fish showed some differential expression of TRs in different tissues. Highest expression was found in the gills and liver tissue, but low basal expression was found in most other tissues studies. Although the differential expression of TRβ in the different tissues of *O. mossambicus* needs more study, clearly TRβ expression was basal in many tissues. The widespread tissue expressions have been reported in other fish, specifically relatively high expression in muscle and gill tissue (Filby and Tyler, 2007). Although in the Japanese eel, expression of TRβ was localized in the brain and pituitary (Kawakami *et al.,* 2007).

In a previous study, estrogen receptor (ER) mRNA expression was investigated during the ontogenic development of *O. mossambicus* and upregulation of ER mRNA occurred early during development (Esterhuyse *et al.,* 2010). The exposure of 20-dpf *O. mossambicus* juveniles to thyroid hormone (T3), for 48 hours, resulted in an increase in expression of TRβ mRNA in whole body homogenates. Although the expression of thyroid receptor (TR) mRNA transcripts have been used widely in amphibian studies, for example after exposing premetamorphic tadpoles to potential thyroid hormones or thyroid disrupting chemicals (Opitz and Kloas, 2010), studies including gene expression responses in fish are

few (Marchand *et al.,* 2001). This study confirmed the upregulation potential of TRβ in 20 dpf juveniles after T3 exposure. Exposing 20-dpf *O. mossambicus* to Bisphenol A (BPA) resulted in the upregulation of TRβ, similar to a study using zebrafish (Chan and Chan, 2012). In mixture with T3, TRβ was upregulated in an added way. In *Xenopus laevis* premetamorphic tadpoles (this study) BPA exposure alone did not have a significant effect on TRβ expression but did result in a significant upregulation when in mixture with T3. In an *in vitro* study, Moriyama *et al.,* (2002) showed that BPA inhibited TR mediated transcription through displacement of T3 and recruitment of a transcriptional repressor that may lead to gene suppression. Recently, Terrien *et al.,* (2011) showed that when exposing transgenic zebrafish (with TR sensitive reporter system) BPA did not modify fluorescence but when tested in mixture with T3 the expected T3-induced fluorescence was reduces significantly, suggesting that BPA interferes with T3 function. Clearly more research is needed to elucidate the BPA mechanism of action in *in vivo* models.

Endosulfan is an organochlorine pesticide extensively used in agriculture on a global scale, but recently being earmarked to be phased out by the USA EPA. This pesticide is also known to be an estrogenic EDC and suggested to also affect development (Grünfeld and Bonefeld-Jorgensen, 2004). Because it is known to be persistent (Dalvie *et al.,* 2003) in the South African aquatic environment, and the bioaccumulation in fish have been shown (Heath and Claassen, 1999) it was included in this study to investigate its potential as a TDC. Endosulfan on its own did not significantly affect the expression of TRβ but when in mixture with T3 did downregulate the expression of TRβ when compared to the expression resulted from T3 alone. Although no study could be found, showing effects of endosulfan on the expression of TRβ in fish published reports suggested that development might be affected when exposed to this pesticide (Silva and Gammon, 2009).

Clearly, assessing the expression of TRβ mRNA in juvenile fish following a short exposure to environmental chemicals, alone or in mixture with thyroid hormone, T3 holds potential as a screening tool. The widespread use of qRT-PCR methodology in toxicogenomics greatly facilitated this advance (Farr and Dunn, 1999).

5 CHAPTER 5: REVIEW OF AVAILABLE *IN VITRO* ASSAYS AS SCREENING METHODS FOR POTENTIAL THYROID HORMONE

5.1 Introduction

The US Environmental Protection Agency (USEPA) responded to a scientific and public concern that a variety of environmental chemicals may disrupt the endocrine system of wildlife and humans (http://www.epa.gov/scipoly/oscpendo/index.htm) and subsequently formed the Endocrine Disruptor Screening and Advisory Committee (EDSTAC) (http://www.epa.gov/endo/pubs/edspoverview/finalrpt.htm) in 1996 to provide a screening programme for EDCs. In the final report, EDSTAC, recognized three main focus areas of potential endocrine disruption resulting from man-made chemicals introduced into the environment, namely, estrogenic, androgenic and the thyroid systems to be included in a two-tiered (tier 1: screening and tier 2: testing) approach. The rationale being that Tier 1 screening tools be designed and validated for rapid detection of potential endocrine disruptors or endocrine disruption activity in environmental samples. Where possible these assays preferably should include *in vitro* (high through-putt) bioassays to limit time-consuming *in vivo* (animal) exposures. This tiered approach has gained international acceptance and the OECD established a working group on Endocrine Disruptor Testing and Assessment to validate and standardize protocols for selected tests (Charles, 2004). In keeping with this objective, the battery of Tier 1 tests selected, include mostly *in vitro* exposures, the only exception being the *Xenopus* metamorphosis assay for thyroid disruption (XEMA) (Degitz *et al.,* 2005; Opitz *et al.,* 2005; Tietge *et al.,* 2005; OECD, 2008a). The complexity of the thyroid system allows for a multiple of potentially effected sites and pathways, increasing the potential of false negatives and excluding the possibility of compensation in thyroid control (Zoeller and Tan, 2007b). However, because of the increasing need for high throughput screening tools and the need to understand the specific modes of action of individual or groups of compounds, the quest to development and validation *in vitro* tests continues. A number of *in vitro* assay have been described in the literature. The assays in use have been developed around the main points of potential influences, namely, thyroid stimulating hormone (TSH), interaction with thyroid follicular cells, iodide uptake by follicular cells, thyroglobolin synthesis and storage, thyroid hormone (TH) transport in the blood, thyroid hormone uptake in target cells, deiodinase enzyme activity (T4 to T3 transformation) in thyroid and target cells, binding of TH to nuclear receptors, regulation of gene expression of TR mRNAs, and metabolism of TH.

Generally *in vitro* assays are not as labour intensive, and of shorter duration when compared to *in vivo* assays. However, these assays can mostly only be applied to investigate specific pathways/endpoints in the complex regulatory network associated with

the thyroid endocrine system. Since the development and validation of *in vitro* tests associated with the thyroid system lags behind development of similar first tier bioassays developed for reproductive endocrine systems, more effort must be put into the validation of endpoint specific *in vitro* assays, although in light of the complexity of the system the use of a battery of tests may seems impractical (Zoeller and Tan, 2007). Although the risk of false negatives will be high when using *in vitro* assays to screen for thyroid disruption (a system with multiple points of interference), early warning signposts remains valuable.

In this review, information on *in vitro* and *ex vivo* bioassays used to screen/test/evaluate chemicals and environmental samples for thyroid disruption were briefly summarized.

5.2 *In vitro* bioassays

5.2.1 Receptor binding assays

It is known that chemicals can alter hormone action by binding to its receptors. Therefore, one of the first assays to be setup and validated in endocrine studies is the classical receptor binding assay. In these assays, competitive receptor binding forms the basis of the assessment. The classical receptor-binding assay for thyroid receptor binding, similar to the estrogen and androgen receptor binding assays, has been attempted (McKinney *et al.,* 1987; DeVito *et al.,* 1999; Date *et al.,* 2002). Several receptor isoforms may be present with differential expression in specific tissues. Nuclear homogenates or TR-isoforms expressed in *Escherichia coli* or specific animal cell lines are used to separate bound from free hormones (Cheng *et al.,* 1994; DeVito *et al.,* 1999; Kudo and Yamauchi, 2005; Kudo *et al.,* 2006; Murata and Yamauchi, 2007). McKinney *et al.,* (1987) and Date *et al.,* (2002) used rat liver homogenates. In solid-state binding assays, TRs are attached to the wall of plates or to beads, which enables the separation of free and bound ligands. Competitive [^{125}I]T_3 binding with or without T3 is performed using environmental samples dissolved in DMSO. The extent to which environmental samples inhibit binding to the TR demonstrates thyroid disruption. Following the separation of free from bound [^{125}I]T_3 the radioactivity is measured in a gamma counter. The amount of [^{125}I]T_3 bound non-specifically is determined from the radioactivity of samples incubated with excess unlabelled T3. This non-specific binding value is the subtracted from the amount of total binding to give specifically bound [^{125}I]T_3 (Murata and Yamauchi, 2007). These binding assays have low potential for false positives but false negatives can occur if metabolic activation is needed or if solubility problems are encountered (DeVito *et al.,* 1999).

In a recent study conducted by Murata & Yumauchi (2007), the TR binding assay was used in conjunction with several other *in vitro* assays and one *in vivo* assay. The TR binding assay performed well in this battery of tests and was regarded a powerful *in vitro* assay to detect thyroid disrupting activity in sewage samples. They concluded that the TR binding assay is suitable for primary high-throughput screening (simple, rapid and cheap to perform), although it does not discriminate between agonist and antagonistic activities of samples . As pointed out by (Zacharewski, 1997), the binding of a substance to the hormone receptor (ER or TR) is only suggestive that it may be an exo-hormone, not providing sufficient evidence of endocrine disruption and that subsequent *in vitro* and *in vivo* tests are necessary to fully assess potential risks.

For the screening of chemicals with a potential thyroid hormone and anti-thyroid hormone activities, transient transactivation assay systems using thyroid hormone receptors (TRα and TRβ) from three frog species (*Xenopus laevis*, *Silurana tropicalis* and *Rana rugosa*), a fish (*Oryzias latipes*), an alligator (*Alligator mississippiensis*) and a human (*Homo sapiens*) in a mammalian cell line (HEK293) have also recently been established (Oka *et al.,* 2012). Similar transcriptional activities were found for all species examined with T3 and T4, with the former inducing activity in 100 fold smaller concentrations.

Several laboratories also combined the *Xenopus laevis* TR assay (see below) with a *Xenopus* laevis TTR assay.

5.2.2 Thyroid Hormone serum carrier protein (thyroid binding globulin, TBG; transthyretin, TTR & Albumin, Alb) binding assays.

These assays can be used to study disruption of the TH (T4 & T3) transport in the blood by the plasma protein carriers (Lans *et al.,* 1994). In humans, TBG is the predominant binding protein, whereas in rodents, TTR is the predominant carrier of thyroid hormones (Richardson *et al.,* 1994; DeVito *et al.,* 1999; Fort *et al.,* 2007). In amphibians, TTR is also the predominant binding protein but albumin is the primary protein transporting TH in certain small mammals, marsupials, birds and reptiles (Richardson *et al.,* 1994; Fort *et al.,* 2007). In contrast to mammals, TH binding proteins have a greater affinity for T3 in lower vertebrates (Chang *et al.,* 1999).

The displacement of thyroid hormone, T4, from the serum binding proteins by environmental chemicals forms the basis of the TTR assay. This assay has been performed widely in several laboratories, mainly focussing on polyhalogenated dibenzo-p-dioxins, biphenyls, and diphenylethers (DeVito *et al.,* 1999). DeVito *et al.,* (1999) pointed out that the displacement of T4 from the carrier protein could have several implications for normal thyroid functioning, for example, increased clearance of T4 and decreased serum T4. Although the

TTR assay have been used extensively and has potential as high-throughput screen, this assay evaluate chemicals that compete with THs for TTR and will therefore only be representative for this mode of action (DeVito *et al.,* 1999). Meerts *et al.,* (2000) and Hamers *et al.,* (2006) used this assay to assess the TRR binding of bromide flame-retardants.

An *in vitro* TTR assay that uses recombinant *Xenopus laevis* TTR (xTTR) expressed in Escherichia coli BL21 cells was recently used to screen for thyroid disrupting activity in domestic sewage treatment plants (Murata and Yamauchi, 2007). The xTTR derived from X. laevis serum has an 80 times higher affinity for T3 than for T4 (Yamauchi *et al.,* 2002). Kudo & Yamauchi (2005) reported that the *in vitro* xTTR assay was more sensitive to chemicals tested than the xTR-binding assay. Moreover, they showed that xTTR and xTR assays had higher affinities for chlorinated derivates of bisphenol A and of nonylphenol than for their parent molecules. Interestingly, they suggest that TRs and TTRs from lower vertebrates preferentially bind phenolic compounds with a T3 like structure, whereas TTRs and TRs from higher vertebrates preferentially bind phenolic compounds with a T4 structure (Kudo and Yamauchi, 2005; Kudo *et al.,* 2006).

5.2.3 Thyroid Receptor binding assay (GH3 cell assay; T-screen)

The GH3 cells, rat pituitary tumor cell line, have been used to detect generalized disruption of TR action analogous to the EScreen for estrogenicity/anti-estrogenicity. GH3 cells have both TRα and TRβ receptors and will proliferate when exposed to T3 (Ghisari and Bonefeld-Jorgensen, 2005; Gutleb *et al.,* 2005; Schriks *et al.,* 2006; Zoeller and Tan, 2007). The growth stimulation is mediated through binding to TRs (either TH or mimics) and the subsequent binding to Thyroid hormone responsive element (TREs) in the nucleus, resulting in increased gene expression (Samuels *et al.,* 1988; Hohenwarter *et al.,* 1996; Schriks, 2006; Schriks *et al.,* 2006). Binding of environmental chemicals to TRs may result in stimulatory effects on cell growth, whereas binding of antagonists to the TRs my result in inhibitory effects on T3 mediated cell proliferation. One of the concerns of this assay is that the effect of estrogenic compounds on cell proliferation is not well understood. Some reports suggest effects of 17β-estradiol on cell proliferation, while others do not. Fujimoto *et al.,* (2004) showed that an anti-estrogen could inhibit T3-induced cell proliferation and that Era mRNA levels in GH3 cells may be upregulated upon T3 treatment. However, Ghisari and Bonefeld-Jorgensen (2005) suggested that the GH3 cells are T3-dependent and that the E2 effect is a secondary sensitivity mediated by T3-dependent ER regulation. They therefore conclude that GH3 cells are T3 dependent but E2 sensitive. The importance of understanding the cross talk between thyroid and estrogen signalling pathways is therefore

underlined. This aspect could be further studied by using the GH3 cells (Ghisari and Bonefeld-Jorgensen, 2005).

One advantage of the *in vitro* GH3-screen is that it uses the whole functionality of cells rather than assessing specific molecules (enzymes and other proteins) involved in specific pathways. In addition, the use of fully functional cells ensures metabolic capability of the cells as well as the complex feedback mechanisms that characterizes the *in vivo* assays (DeVito *et al.,* 1999). A concern mentioned by Schirks (2006) is the fact that the GH3 cells do not have CYP-activity, which may result in false negative responses when certain compounds need bio-activation in the *in vivo* system. It was therefore suggested that a prior biotransformation step be included.

The T-screen (GH3 cell assay) has been used in several studies to screen for disruption of TR action in a similar manner as the E-screen (Zoeller and Tan, 2007). Several classes of environmental chemicals have been screened using this assay, including plasticizers, alkylphenols, pesticides, PCB metabolites and brominated flame retardants) (Ghisari and Bonefeld-Jorgensen, 2005; Hamers *et al.,* 2006; Schriks, 2006; Schriks *et al.,* 2006).

5.2.4 FRTL-5 cell assay

Other *in vitro* assays may be aimed at specific pathways; for example, FRTL-5 cells (Fisher rat thyroid line) have the ability to concentrate iodide (Ambesi-Impiombato *et al.,* 1980; Brown *et al.,* 1986). The FRTL-5 cells characterize fully metabolic active thyroid cells and can be used to screen for effects of environmental chemicals, specifically iodide uptake mediated by the sodium/iodide symporter (NIS) (Wenzel *et al.,* 2005) and the activity of thyroid peroxidase (TPO) enzymes (Zoeler and Tan 2007). Plasticisers, dialkyl phalates, have been shown to modulate iodide uptake, several enhancing iodide uptake (Wenzel *et al.,* 2005).

Thyroid peroxidases (TPOs) are enzymes in the synthesis pathway of thyroid hormones (TH). These enzymes play a role in the iodination of tyrosine and coupling of specific di- and triiodotyrosyl on thyroglobulin. Several chemicals (including propylthiouracil (PTU), Methimazole (MTI), perchlorate) are known to directly interfere with TPO activity (Wolff, 1998; Wenzel *et al.,* 2005). The thyroid peroxidase assay using FRTL-5 cells can therefore, be used to test for the ability of chemicals to block the activity of this enzyme (Wenzel *et al.,* 2005; Zoeller and Tan, 2007b).

Marinovich *et al.,* (1995) inserted a full-length cDNA clone for human thyroid peroxidase in an expression vector and stably transfected it into the FRTL-5 cells. The

effects of toxicants on human TPO activity can be studied with these cells. They demonstrated that MTI (antithyroid agent) and ethylenethiourea (a metabolite of dithiocarbamate pesticides) inhibited TPO activity in a dose-dependent matter.

FRTL-5 cells can also be used as proliferation assays, but the pretreatment of FRTL-5 cells with TSH is necessary to facilitate the mitogenic response (Park *et al.,* 2008). Thyroid peroxidase mRNA and thyroglobulin mRNA have been used as biomarkers after Methimazole (MTI, antithyroid agent) and propylthiouracil exposure (Leer *et al.,* 1991).

5.2.5 Chinese hamster ovary cells (CHO)

Chinese hamster ovary cells (CHOs) transfected with recombinant human TSH receptor are also used to study the TSH-induced adenylate cyclase activation and cAMP production (Santini *et al.,* 2003). This represents the first steps of thyroid gland activation, following the binding of TSH to a specific receptor on the cell surface. The activated receptor interacts with G-proteins that in turn interacts with and activates adenylate cyclase, which catalyzes the production of cAMP, playing a pivotal role in pathways associated with most thyroid functions (Santini *et al.,* 2003). Therefore, the CHO bioassay can be used to identify exogenous substances capable of interfering with TSH-induced cAMP production. Santini *et al.,* (2003), using several assays associated with *in vitro* exposures of the CHO cells, showed that extracts from the lemon balm herb, Melissa officinalis, Aroclor 1254, a mixture of polychlorinated biphenols (PCBs) and DDT, an organochlorine insecticide used to combat malaria, may interfere with the TSH-receptor interaction or are TSH-induced adenylate cyclase inhibitors.

Chinese hamster ovary cells (CHOs) have also been used to study the inhibition of the sodium iodide symporter (NIS). Several studies report the use of a CHO cell line stably transfected with human NIS to study the effects of compounds on the NIS system (Tonacchera *et al.,* 2004). Normally, radioactive iodide uptake (RAIU) inhibition and radioactive iodide (RAI) discharge are used to evaluate disruption of the NIS. Since a number of dietary or environmentally relevant inorganic anions, for example, nitrate (NO_3^-), thiocyanate (SCN^-) and perchlorate (CLO_4^-) can block iodide uptake, decreased thyroid hormone production could lead to adverse health effects (Tonacchera *et al.,* 2004). Three studies using the NIS transfected CHO cell lines demonstrated inhibition of RAIU in a dose-dependent manner when exposing cells to CLO_4^-, SCN^- and NO3-, CLO_4^- being the more potent inhibitor (Greer *et al.,* 1966; Tonacchera *et al.,* 2001; Tonacchera *et al.,* 2004).

5.2.6 Reporter Gene Assays

CV-1 cell line: – This is a green monkey kidney fibroblast cell line transfected with either *Xenopus* TRα or TRβ and a BTEB Thyroid response element (TRE) luciferase reporter plasmid and pCS2-β-galactosidase. Following exposure, cells were lysed and assayed for luciferase and β-galactosidase activity (Furlow *et al.,* 2004). Schirks *et al.,* (2006) used this assay to screen brominated diphenylethers and halogenated hydrocarbons for changes in thyroid receptor expression (TRα & TRβ). T3 induced luciferase induction for both TRs occurred in a dose-dependent manner. The EC50 for TRα activation by T3 was three times lower than the EC50 for TRβ activation. Their results corresponded well with T-screen results, but they emphasized the importance of testing potential thyroid disrupting activity of compounds using the expression of both TR isoforms

XL58-TRE-Luc cells: – Sugiyama *et al.,* (2005) reported on the development of a reporter gene assay in which they used a lentivirus vector to transfect a *Xenopus laevis* cell-line (XL58). The lentivirus contains a luciferase gene downstream from the *X. laevis* T3 response elements (TREs) (TRβ-1). The resulting recombinant *X. laevis* cells (XL58-TRE-Luc) were validated and used in a reporter gene assay to screen chlorinated derivatives of Bisphenol A. Of the six chemical tested they found that only one (3,3,5-trichlorobisphenol A) inhibited the T3 dependent luciferase activity. Murata and Yamauchi (2007) used the same assay to screen for thyroid system-disrupting activity in effluents of domestic sewage treatment plants. From this study they concluded that the Luc assay could be a powerful first tier assay for high-throughput screening. The Luc assay can discriminate between agonist and antagonist activities in samples.

PC12 cell line: – Recently, Jugan *et al.,* (2007) developed a TRα-1 mediated assay based on the stable transformation of a luciferase reporter gene in PC12 mammalian cells, designed to express TRα1 (Munoz *et al.,* 1993). The alpha (α) isoform of TR is of avian origin. The validated this assay and used it to screen several halogenated phenolic and phenol compounds suspected of being thyroid-disrupting environmental contaminants. Jugan *et al.,* (2007) have suggested that this assay can be used in high-throughput situations and could detect agonistic and antagonistic activities. Because they used four consecutive repeats of the optimal TRE motif, they have reported a 15 fold induction of T3 in this TRα1-expressing PC12 mammalian cell line compared to the three-fold induction of the luciferase in response to T3 reported by Sugiyama *et al.,* (2005), using the *X. laevis* cell line expressing the TRβ-1 receptor.

5.2.7 Recombinant Yeast cells

Transformed yeast cell lines containing TR gene constructs have been developed, showing differential transcription through increased enzymatic activity of the reporter gene product (for example, luciferase or β-galactosidase) (DeVito *et al.,* 1999). A yeast two-hybrid system was developed to test for binding of chemicals on human thyroid receptors (TRs) (Nishikawa *et al.,* 1999; Kitagawa *et al.,* 2003). This initial yeast assay included TRα and the co-activator, transcriptional intermediary factor 2 (TIF-2). The β-galactosidase reporter gene is also included, transformed yeast cells are then incubated with control compounds (for example T3), chemical compounds or environmental extracts at 30ºC for 24 hours. Binding to the TR correlates with galactosidase expression and is measured spectrophotometrically. Agonistic and antagonistic activities are assessed by incubating compounds or samples alone and in combination with T3. The dose-dependent binding to TR and therefore dose-depending expression of b-galactosidase was confirmed at concentrations as low as 3×10^{-8} M T3 (Kitagawa *et al.,* 2003). Kitagawa *et al.,* (2003) used a 10% relative effective activity (REC10) to evaluate relative activity. REC10 represents the concentration of the test chemical showing 10% of the agonist activity of 1.0×10^{-6} M T3, the optimum concentration they reported for T3. They judged a sample to be positive if the activity exceeded the 10% REC.

Li *et al.,* (2008) developed a similar yeast assay, making use of recombined TR (TRβ) and the co-activator, GRIP1 but also using the β-galactosidase reporter gene to indicate activation through binding with TRs. They use 5×10^{-6} M T3 concentration to assess antagonistic activity. In contrast to Kitawaga *et al.,* (2003), Li *et al.,* (2008) used a relative effective concentration of 20% (i.e., 20% of the maximum effect or reducing 20% of the maximum effect) rather than 10% as indicator of compounds showing effects. They also showed that this yeast assay system is highly specific and that there is little cross talk to other steroid receptors. In the absence of TRβ transfection, β-galactosidase activation did not exist, therefore confirming that T3 was not able to give a β-galactosidase response in the absence of TRβ. Li *et al.,* (2008) tested 19 chemicals, including a series of phenols, phthalates, organochlorine pesticides, poly-halogenated aromatic hydrocarbons as well as known agonists (T4) and antagonists (amiodarone). They found 15 of the 19 xenobiotic compounds to be antagonists (decreasing normal ligand, T3, binding) and the other to be agonists, thus providing additional ligands to bind TRs.

Although the recently developed TR-specific yeast assays have been validated and shown great potential for high through-put screening (Kitagawa *et al.,* 2003; Li *et al.,* 2008), disadvantages include, the suspected limited entry into the yeast cell because of the cell wall

and the lack of metabolic capabilities of these cells. Therefore, if it is the metabolite of the compound, rather than the parent compound that binds to the receptor, then a false negative could be the result. Similar to other comparative studies evaluating recombinant yeast assays against animal cell proliferation or recombinant reporter gene assays transfected with steroid receptors (for example, ERs) (Leusch, 2008), the performance of the recently validated recombinant TR yeast assay should be evaluated against other animal cell-based TR assays.

5.2.8 Ex vivo bioassays

5.2.8.1 Thyroid slices in culture

Although few studies have pursued the *ex vivo* culturing of thyroid slices to study thyroid related responses, previous studies have shown that *ex vivo* culturing of organ slices, for example liver (Hurter *et al.,* 2002) can be effectively used to study specific responses associated with hormonal and xenobiotic exposures. Shuman *et al.,* (1976) exposed thyroid slices to study the response of the cyclic AMP system after TSH exposure. In a recent study, *Xenopus laevis* thyroid explant cultures were evaluated for thyroid disruptor screening. Hornung *et al.,* (2010) showed that thyroid explants could be cultured successfully and provide an alternative *in vitro* system for assessing the potential of chemicals to disrupt normal TH production and release. They showed that the explants responded to TSH in a dose related manner for T4 release and that inhibitors of TH synthesis could also be identified. Histological analysis of the explanted thyroid glands after eight days showed that the glands are a robust system in culture and they concluded, that although more work is needed to validate this assay, it may be a good first tier screen, to get an initial simple yes or no answer regarding the capacity of a chemical to alter TSH-stimulated TH release, but they were confident that this assay could also be used to do a full dose-response study to compare relative potencies of known TH disruptors (Hornung *et al.,* 2010). Therefore, it seems that use of ex vivo thyroid slices to study other aspects of thyroid function holds great potential as high through-put assay and may be less labour extensive than the 21-day XEMA.

5.2.8.2 The African Clawed frog, *Xenopus laevis*, tails in culture

Several studies also report *ex vivo Xenopus* tail tissue culture assays (Tata, 1966; Iwamuro *et al.,* 2003; Furlow *et al.,* 2004; Iwamuro *et al.,* 2006; Schriks, 2006). In this assay the tails of Xenopus laevis tadpoles (NF-stages 52-54) (Nieuwkoop and Faber, 1956) are removed surgically and placed in culture. The tails are then exposed to different compounds including control hormones and measured every 24 hours for a period of four days or six days (Schriks, 2006). The rationale being that TH induces regression of the tail and substances

could delay or increase the regression process. Correspondence between ex vivo tail resorption studies and other *in vitro* assays (for example T-screen and TR mRNA expression) were good in both studies (Iwamuro *et al.,* 2003; Iwamuro *et al.,* 2006; Schriks *et al.,* 2006).

5.2.8.3 Amphibian red-blood cells: disruption of transporter proteins

Recently the suggestion was made that certain EDCs may disrupt the cellular uptake of TH (Yamauchi and Ishihara, 2006; Crofton, 2008). Although initially believed that the TH enters the target cell simply by diffusion, recent work has shown that specific TH uptake systems in the cell membrane may exist (Abe *et al.,* 2002; Ritchie *et al.,* 2003; Yamauchi and Ishihara, 2006). At least three groups of thyroid hormone uptake transporter proteins have been suggested. Yamauchi and Ishihara (2006) pointed out that in spite of this knowledge; few studies address the possibility of EDCs affecting the uptake and export of TH over the cell membrane. In this regard, Shimada & Yamauchi (2004) used the red blood cells of the frog (tadpoles), *Rana catesbeina* to investigate the possible effects on TH uptake in the presence of a variety of inhibitors and potential competing amino acids. With this *in vitro* system and preliminary results, they suggest that red blood cells could be candidate target sites for EDCs to modulate the cellular T3 response.

5.3 Conclusion

Although the complexity of the thyroid endocrine system limits the use of single *in vitro* assays as first tier screens for endocrine disruption of the thyroid system, several endpoint-specific *in vitro* assays have been developed and validated (DeVito *et al.,* 1999; Schriks, 2006; Zoeller and Tan, 2007; Crofton, 2008). Therefore, the *in vivo* exposure of developing tadpoles (*Xenopus* Metamorphosis Assay) as bio-indicators, including disruption at any of the multiple possible endpoints in several associated pathways, is preferred as first tier screen (Tan and Zoeller, 2007). However, the demand for short-term, fast screening at first tier levels made researchers combine several endpoint-specific *in vitro* assays with the XEMA test (Iwamuro *et al.,* 2003; Kudo and Yamauchi, 2005; Kudo *et al.,* 2006). This allows for answering questions regarding specific modes of action alongside whole-body thyroid effects (through the XEMA approach). Combinations of *in vitro* assays also allows for investigating multiple effects of environmental chemical compounds (Murata and Yamauchi, 2007). The future of putting together a battery of *in vitro* (*ex vivo*) screens to be used in high throughput situations therefore holds great potential and need urgent attention. Although very little screening for thyroid disruption has been attempted in South Africa, it is envisaged

that it is only a matter of time and *in vitro* / *ex vivo* bioassays will be used for screening water samples and selected man-made chemicals with potential thyroid disrupting activity.

6 PROJECT CONCLUSIONS

Reviewing the thyroid disruption literature base showed that a knowledge explosion is currently happening in this field. This is partly the result of the concern that thyroid disruption caused by contaminants in the environment, including the aquatic environment is a major EDC threat, directly related to developmental programmes as well as controlling metabolic pathways. In addition the cross-talk of the thyroid system with other endocrine systems underlines the importance of the thyroid endocrine system and the wide-reaching effects that disruption of this system may have when targeted by thyroid disruptor compounds (TDCs).

The development and validation of the *Xenopus* metamorphosis assay (XEMA) was an important development and although a relative long and laborious assay to conduct, it is a good assay to assess how the whole thyroid system response to a thyroid disrupting contaminant (of which the specific mechanism of action may still be unknown). Using a semi-static set-up permits for a reasonably simple exposure system to screen for potential thyroid disrupting activity (specifically TH mediated). Morphological and developmental endpoints are well developed. More research is needed to link histopathological changes to endocrine disrupting activity. The use of short-term TR expression bioassays, involving RT-QPCR analysis of TRβ mRNA, holds great potential as an indirect bioassay. Several *in vitro* bioassays are available and used only by a few researchers to address specific questions regarding certain modes-of-actions/endpoints in the complex thyroid endocrine system. Clearly, this field will rapidly expand in the near future.

Although one of the main aims was to establish working bioassays locally, reviewing the literature and summarizing the advances made recently in screening environmental chemicals and aquatic samples for TDC activity clearly was an important secondary aim. From a bioassay perspective, we asked the question of whether we are ready to initiate screening programmes that will address endocrine systems other than the reproductive systems. All the aims were reached, except for the development of additional biochemical endpoints associated with the extensive thyroid endocrine system and the possible incorporation thereof into specific bioassays. Clearly, more research is needed in this regard.

One of the benefits/outcomes of this project is the fact that it confirmed that we have the capacity in South Africa to do assessments for thyroid endocrine disruptors. The project will also benefit the managers and researchers since they will gain background information in the potential of thyroid disruption by contaminants in our water resources (a field largely shadowed by reproductive disruption). Since we succeeded in validating the XEMA assay

we also present basic procedures and design approaches to setup and conduct the XEMA. In addition, this project for the first time, also introduces the idea of extending EDC biomarkers to the molecular level. Here we successfully, demonstrated the potential of using juveniles of two local freshwater species, tadpoles of the African clawed frog, *Xenopus laevis* and juveniles of the Mozambique tilapia, *Oreochromis mossambicus* in short-term thyroid receptor binding assays. Valuable information on the potential application of quantifying specific thyroid receptor gene expression and the effects of aquatic contaminants on these genes is presented. It is therefore clear that at several levels and in several areas related to endocrine disruption in South Africa, this project has expanded capacity and should the screening and testing for thyroid disruption be initiated on a national scale, we now have the capacity to implement XEMA and short-term molecular assays.

7 RECOMMENDATIONS

1. Screening assays for thyroid disruption should, as a matter of urgency be used more widely, at least in water treatment laboratories.
2. Training workshops should take place to train researchers to use the XEMA testing approach, including the in house breeding of breeding *Xenopus* laevis tadpoles.
3. Training workshops in data analyses and thyroid histopathology should be held.
4. Research towards refining the XEMA testing by reducing the period of exposure and conducting the exposure in the early pro-metamorphosis phase of development.
5. Research on the set-up, development and validation of specific *in vitro* assays related to the most important points of disruption should continue.
6. Expand the molecular endpoints (mRNAs) and use of RT-QPCR in short-term exposures of tadpoles and juvenile fish.
7. Research and establish the epidemiology of thyroid related diseases in South Africa that specifically link to environmental contaminants
8. Future research should target and assess human activities known to produce compounds that potentially may disrupt the thyroid systems of wildlife and humans.
9. Initiate research to develop basic and ecological risk assessment models that specifically include thyroid disruption as an important risk factor.

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