The interaction of chemicals isolated from municipal wastewater effluent with rainbow trout (*Oncorhynchus mykiss*) thyroid hormone receptors

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2012

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

The normal function of the thyroid hormone (TH) system is essential for growth, development and metabolism in humans as well as in other species. The action of TH is dependent on its binding to thyroid hormone receptors (THR) found in the cell nucleus. In some situations, chemicals with structural similarities to TH can bind to these receptors and disrupt their normal function. It has been previously demonstrated that environmental contaminants including, carbamazapine, nonlyphenol (NP), bisphenol A (BPA), and several others are able to bind to the THR as either agonists or antagonists and modulate downstream biochemical responses. Municipal wastewater effluent (MWWE) is a major source of these contaminants entering aquatic environments. Recently extracts of MWWE have been shown to contain chemicals that are capable of binding to THRs. However, MWWE is a complex mixture of chemicals and the specific chemicals have not been identified. In this thesis, a proof of concept was developed for using an Effects Directed Assessment (EDA) approach to isolate thyroid receptor active compounds in MWWE. An EDA is a technique created to extract and identify chemicals from complex mixtures, using various fractionation methods. Once these chemicals have been identified, they are further reviewed for biological relevance. A competitive binding assay for THR was developed and applied to determine the relative binding affinity of known environmental contaminants to THR. Nuclear thyroid hormone receptors were isolated from rainbow trout liver by differential centrifugation. This method involved liver tissue homogenization and subsequent centrifugations to separate the nuclear fraction containing the receptors. The binding characteristics of the isolated THR were evaluated using the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) in a competitive binding assay. Minimal binding affinity was present in this assay and future studies should validate the assay further and assure that it is comparable to literature values. Environmental contaminants, including BPA, NP were also tested to determine their relative binding affinity to the THRs compared to the endogenous hormones. High concentrations of both BPA and NP bound to the thyroid hormone receptor, displacing radiolabeled T_3 from its binding site. The

rainbow trout competitive binding assay was also used to test the binding affinities of extracts from two municipal wastewater effluents collected in the Grand River watershed in southern Ontario. Effluents were extracted using a solid phase adsorbent (HLB Oasis cartridge), eluted with methanol, taken to dryness then reconstituted in ethanol for use in the assay. Both effluent extracts displaced the binding of radiolabeled T_3 to the thyroid receptors. The studies demonstrate that a competitive THR assay can be used to detect chemicals in complex mixtures with the potential to interact with THRs. The next step should be to apply the assay using an EDA approach to isolate and identify specific chemicals in effluents that are not yet known to bind to the THR. Interference with the normal function of the TH system has the potential to disrupt normal growth, development and metabolism in aquatic organisms in the receiving environments.

Acknowledgements

Dedication

I would like to dedicate my thesis to my parents and my brother who have supported me through all my endeavors and my friends and family who have been patient with me throughout my educational career.

Table of Contents

List of Figures

ix

List of Tables

Chapter 1
Introduction

The thyroid hormones (TH), T4 and T3 are found throughout the body in animals where ; it plays they play a key role in maintaining normal physiological function through growth, development and behavior (Sholz & Mayer, 2008). The synthesis of TH is dependent on thyroglobulin, which is an important precursor protein produced by the endoplasmic reticulum of cells (Bently, 1998). Regulation of TH synthesis and release originates at the level of the brain, more specifically, the hypothalamic-pituitary-thyroid (HPT) axis (Blanton & Specker, 2007). Beginning at the hypothalamus, chemical signals trigger the release of hormones such as, thyrotropin releasing hormone (TRH) and thyroid stimulating hormone (TSH) from the pituitary. Both are linked to the release of predominantly L-thyroxine (T_4) from the thyroid gland where it is synthesized as part of the precursor protein thyroglobulin. (Eales and Brown, 1993). The active thyroid hormone T_3 is synthesized from T_4 in the peripheral tissues by dieodination.   In most fish species, the thyroid gland demonstrates greater heterogeneity than in mammals, containing both follicles and cells of differing sizes as well as functional states (Eales, 1979). Mammalian thyroid glands are usually compact and located at the anterior aspect of the neck, whereas, fish thyroid glands are more variable in location and size. Follicles in fish are widely distributed throughout the connective tissue near the pharyngeal regions, they can be observed around the eye, ventral aorta, hepatic veins and anterior kidney (Moeller, 2007; Blanton and Specker, 2007). Thyroid gland variation can also occur between and within fish taxa.   As blood plasma levels of T_3 and T_4 rise, transport proteins facilitate the movement of THs across the liver, brain and peripheral tissues (Jugan et al., 2010; Blanton & Specker, 2007). Once elevated levels have been detected, a

negative feedback signal is sent back to the brain to prevent further hormone synthesis (Figure 2)

(Boas et al. 2006; Pease & Braverman 2009; Blanton & Specker, 2007). In this way, the thyroid

cascade is tightly regulated; however, disruption of TH availability, signaling or transport could

negatively impact the system and hinder integral physiological processes (Moriyama et al.,

2002). Exogenous compounds responsible for this are commonly classified as endocrine

disruptors. These compounds modify natural endocrine function by interacting at different levels

of the thyroid system. This includes direct interaction with receptors, thyroid gland stimulation,

TH synthesis, metabolism, and transport. Endocrine disruption is not solely linked to receptor

interference; other areas within a regulatory network can be linked to endocrine system

disruption (Moriyama et al., 2002; Jugan et al., 2010). To test for disruption, a number of in vitro

bioassays have been developed to determine the ability of chemicals to disrupt the HPT axis

function. In vitro bioassays have been further applied to an Effects Directed Assessment (EDA)

to isolate and identify chemicals in complex mixtures that can potentially impact this axis.

Bioactive fractions from extracts are applied to bioassays (at higher levels of organization) to

understand and verify the potential negative impacts caused by environmental contaminants

(Hecker & Hollert, 2009). This approach can be beneficial in pin- pointing which fractions and

compounds are responsible for causing endocrine disruption in animals, facilitating risk

assessment.

1.1 Physiology of the Thyroid System

The thyroid system is a fundamental component of all animals, and environmental

contaminants may play a role in altering their function and potentially impacting overall health

of exposed organisms. This can occur by altering thyroid hormone homeostasis, which plays a key role in vertebrate metabolism, growth and development (Power et al., 2001; Kashiwagi et al., 2009). The main focus of this chapter is to understand pertinent physiological processes within in the thyroid hormone cascade, locate areas that have shown vulnerability to endocrine disruptors and to identify previous research using in vitro bioassays to determine mechanisms of action. This chapter therefore supports the selection of endpoints and applications of an EDA for complex mixtures.

1.2 Thyroid Gland

Thyroid glands contain thyroid follicles, which are the site of thyroid hormone production (Boulton et al., 1986; Evans, 1988). Thyroid follicles are the functional unit of the thyroid gland in vertebrates and consist of epithelial cells known as, thyrocytes or follicular cells (Bently, 1998). Thyroglobulin is a glycoprotein synthesized in the endoplasmic reticulum of the follicular cells and is secreted into colloids which are enclosed extracellular spaces, forming the lumen. Thyroid follicles interact with inorganic iodide from the blood, which is integrated into tyrosine residues within thyroglobulin (Bently, 1998). Thyroglobulin is a precursor to thyroid hormones; when oxidized by the thyroid peroxidase (TPO) enzyme it forms L-thyroxine, or T_4. TPO is localized in the apical cytoplasmic membrane of thyroid epithelial cells and its main role is T_4 biosynthesis (Schmutlzler et al., 2007). Biosynthesis of T_4 begins with adenosine triphosphate (ATP) driven Na/ I^- symport (NIS, I^- pump), which concentrates iodine (I^-) in the blood and facilitates its diffusion into the follicle lumen. Thyroglublin is then secreted into the follicle where tyrosyl residues within TG are oxidized by TPO to form either a monoiodotyrosyl

3

(MIT) or diiodotyrosyl (DIT) residues. Paired DIT's couple covalently through an ether bond to form tetraiodothyronly residues, also known as T_4; this form is still coupled with TG and stored in the colloid. The apical cell membrane mediates endocytosis of the thyroglobulin T_4 complex. Phagolysosomes contain hydrolytic enzymes responsible for inducing proteolysis, causing the separation of T_4 from the TG and preparing it for transport into the blood (Brown et al., 2004). The prohormone T_4 is required for the synthesis of 3,5,3'-triiodo-L-thyronine (T_3), the active thyroid hormone. The conversion of T_4 to T_3 generally occurs in the peripheral tissues such as the liver (Eales & Brown, 1993).

3,3',5-Triiodothyroxine (T_3)   3,3',5,5'-Tetraiiodothyroxine (T_4)
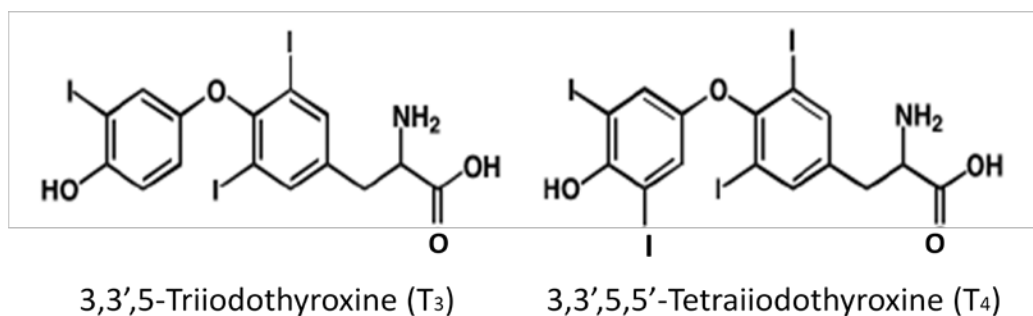
Figure 1 Structure of thyroid hormones T_3, T_4 (Kashiwagi et al., 2009)

1.3 Hypothalamic-Pituitary Axis

The HPT axis is primarily responsible for the regulation of TH synthesis and release. TRH is a tripeptide secreted by the hypothalamus and it functions to regulate the release of TSH from the anterior pituitary (Bently, 1998). Control over thyroid hormone release is regulated by the negative feedback effect on TSH secretion by the pituitary (Yoshiura et al., 1999). Regulation of thyroid hormone helps manage the levels of T_4 and T_3 that are present in the tissues. TSH released from the pituitary then acts on thyroid follicle where it is responsible for T_4

release and iodine uptake. Iodide availability is extremely important in thyroid system

functioning because of its role in the production of thyroid hormones (Eales & Brown, 1993).

This mechanism can be seen in

(Figure 2).
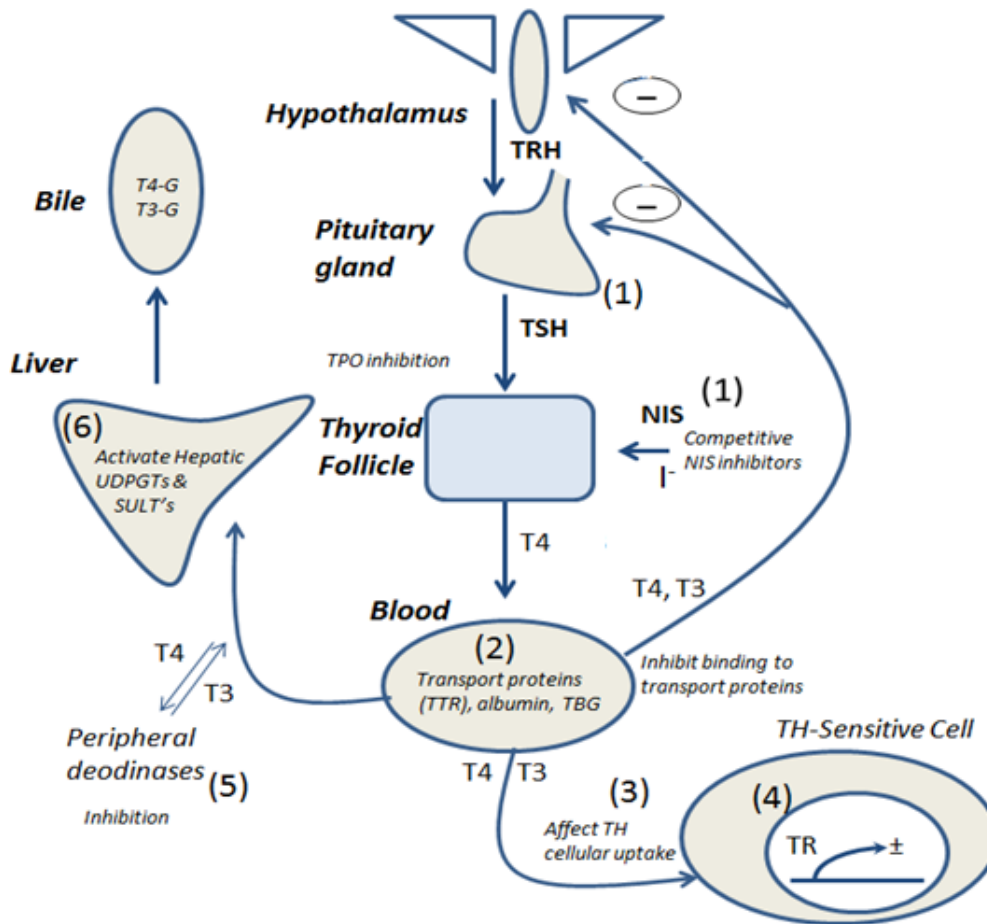
Figure 2 Possible mechanisms by which environmental chemicals could affect the hypothalamic-
pituitary-thyroid axis: 1. Synthesis of thyroid hormones (TH), interference with sodium iodide
symporter (NIS), thyroperixidase (TPO) or thyroid stimulating hormone TSH, 2. Transport
proteins, 3. Cellular uptake mechanisms, 4. Thyroid receptor, 5. Iodothyroninedeiodinases, 6.

Metabolism of TH in the liver; TRH- thyrotropin releasing hormone, T_4-thyroxine, T_3-triiodothyronine, I^- - iodine, UDPGT - diphosphateglucuronyltransferase, TR - thyroid hormone receptor (adapted from Boas et al. 2006, and Pease and Braverman, 2009).

1.4 Thyroid Hormone Activation and Deactivation

Deactivation of thyroid hormone occurs when the iodine atom is removed from the inner ring, this is called inner ring deiodination (IRD). Conversion of T_4-IRD produces reverse T_3 (rT_3 3,3'5 triiodothyronine), T_3 can then further be degraded by inner ring deiodination to inactive 3,3' triiodothyronine (T_2) (Eales et al., 1999, Brown et al., 2004). Outer ring T_4 deiodination (T_4-ORD) forms T_3, a more active form of TH. Deiodinases are enzymes responsible for converting T_4 to T_3 (Brown et al., 2004). There are three deiodinases D1, D2, and D3 (5). D1 is expressed primarily in the liver and regulates systemic TH levels. It has the ability to activate and inactivate TH by mediating ORD and IRD of T_4 and T_3 (Qatanani et al., 2005). D2 is an enzyme that only catalyzes the ORD of TH and D3 is an inactivating enzyme that only catalyzes IRD of TH (Qatanani et al., 2005).

Conjugation involves the glucoronidation and sulfation of the phenolic hydroxyl group. This is important because it increases water solubility and allows for urinary and biliary clearance (Burchell & Coughtrie, 1989; de Herder et al., 1988). Inactivation of TH specifically T_3, is facilitated by sulfation because IRD of sulfated T_4 and T_3 is enhanced 40-200 fold by D1 and the ORD of sulfated T_4 is blocked (Visser, 1994). The nuclear hormone receptor constitutive androstane receptor (CAR) mediates the induction of hepatic drug metabolism in response to xenobiotics and endocrine disruptors. Drug transporters and metabolizing enzymes are UDP-gluuronosyltransferases (UGTs) and sulfotransferases (SULTs). Hepatic UGT and

SULT are also responsible for eliminating THs. CAR was shown to mediate several isoforms of UGTs and SULTs involved in glucuronidation and sulfation of THs (Maglich et al., 2004). Therefore it has been shown that activation by xenobiotics or endocrine disruptors can result in disruption of TR activity (Qatanani et al., 2005).
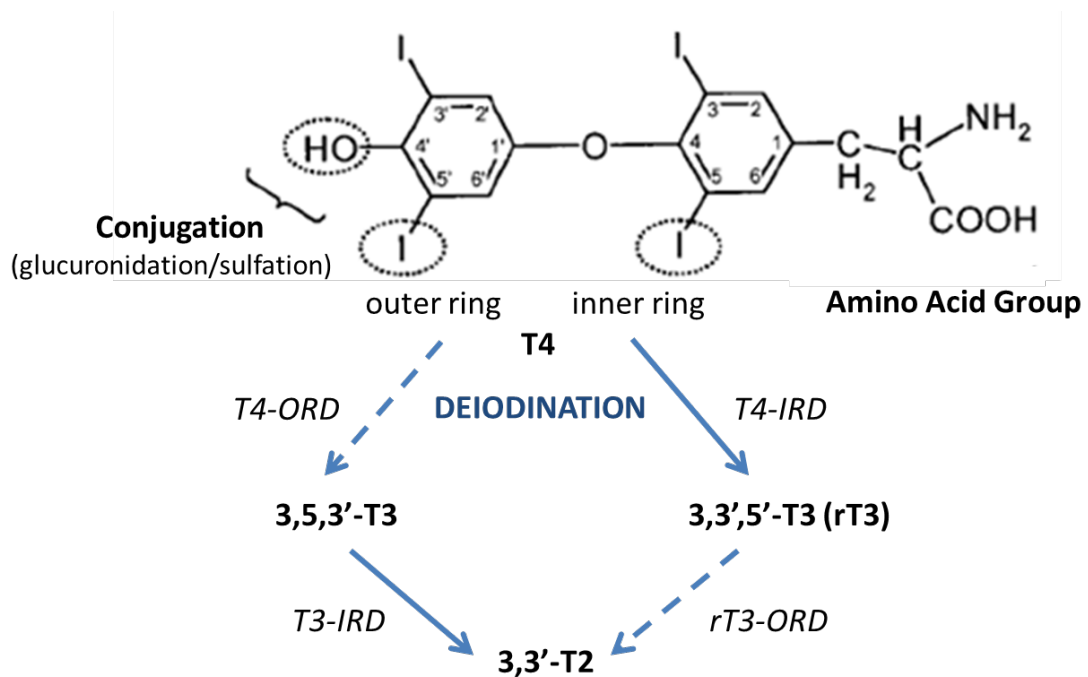
Figure 3 Deiodination pathways L-Thyroxine (T_4) demonstrating where deiodination occurs. ORD refers to the outer ring deiodination, IRD is inner ring deiodination (adapted from Brown et al., 2004).

1.5 Thyroid Hormone Transport

Once the TH is released from the thyroid follicle it needs to be transported to the blood. At physiological pH (approximately 7.0, (Wang et al., 2011)); both T_3 and T_4 are poorly soluble

in blood and are bound to plasma proteins such as thyroid binding globulin (TBG), transthyretin (TTR) and albumin. The binding proteins ensure a constant supply of TH to cells and tissues while preventing loss to the environment, protect the organisms from rapid changes in the TH production and degradation and prevent iodine deficiency (Rasmussen & Rasmussen, 2007). These three proteins are synthesized in the liver and also released into the blood where they are able to bind the THs and distribute them to different cells and tissues throughout the body. With respect to TH affinity, TBG has the highest followed by TTR and lastly albumin (Robbins & Edelhoch, 1986). TTR has been identified as the only TH binding protein from the three mentioned to also be synthesized in the brain (Schreiber, 2002) where it is a major thyroid hormone binding protein in cerebrospinal fluid responsible for moving TH from the blood across the blood-brain barrier into the brain (Schreiber, 2002). Therefore although it has lower affinity than TBG it is still critical for several thyroid related functions. Albumin is a large liver protein that binds to a variety of important hormones, fatty acids and other hydrophobic compounds (Peters, 1985). Albumin only consists of few sites where T_4 and T_3 can bind with high affinity; as a result, only 5% of all serum T_3 and T_4 can be transported by albumin, making it a lower affinity thyroid hormone binding protein (Refetoff et al., 1972; Hollander et al., 1968).

1.6 Thyroid Hormone Receptors

Thyroid hormone receptors have several different isoforms; these gene transcripts are expressed ubiquitously throughout the body, with the α and β isoforms having distinct tissue specificity (Yamano & Miwa, 1998). The similarity between isoforms allows for the binding of both to T_3 and T_4. However, the expression pattern of each isoform varies in a tissue specific TH

response (Furlow et al., 2004). TRα1 is predominantly found in the brain, heart and immune system, whereas, TRβ1 is expressed in the brain, liver and kidney (Jugan et al., 2010). Receptors are required for the activation or deactivation of several molecular processes. Nuclear receptors are transcription factors that distinguish between specific sequences in target genes by way of the DNA binding domain (Evans, 1988). Receptors can bind to DNA as monomers, homodimers or heterodimers. Dimerization is the physical interaction between related proteins and it is often required for the activation of the cell signal. Dimerization and binding to receptors, allow for the transfer of cell signals from the cell surface to the nucleus (Klemm et al., 1998). The mechanism of action varies depending on the type of receptor activation that takes place. Dimerization can either occur in the cytosol or in the nucleus. In each case, the ligand binds to the dimer and causes the recruitment of several proteins leading to receptor activation and upregulation of target gene expression and ultimately changes in cell function. Type one receptors require the ligand to bind to the dimerized receptor complex in the cytosol and then diffuse into the nucleus, while type two receptors require the ligand to diffuse into the nucleus prior to binding to the dimer (Olefsky, 2001). Nuclear receptors are modular in structure they contain the DNA binding domain (DBD), ligand binding domain (LBD) and the amino terminal domain (NTD) (Evans, 1988). The DBD is responsible for binding to specific sequences on the DNA, known as the response element. The LBD is responsible for dimerization of the nuclear receptors (Shao & Lazar, 1999). The amino terminal domain (NTD) contains the activation function 1 (AF1) which is activated independent of a ligand. Activation function 2 (AF2) is found in the LBD, and it works in synergy with AF1 to promote transcription of the DNA (Olefsky, 2001).
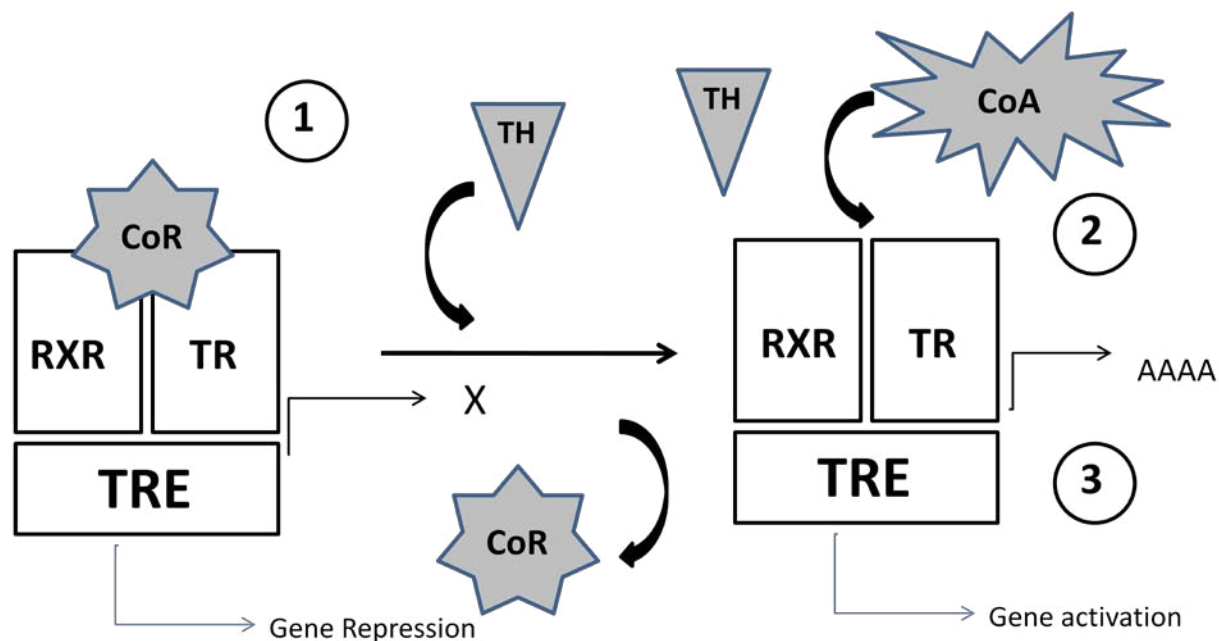
Figure 4 Regulation of thyroid hormone receptor (TR) binding. 1) In the absence of a ligand, a corepressor is bound to the thyroid hormone receptor and prevents transcription of the downstream genes. 2) However, once a ligand binds to the thyroid hormone receptor the corepressor dissociates resulting in the recruitment of the coactivator protein along with several other proteins such as, RNA polymerase. 3) This allows for transcription of DNA into mRNA which is subsequently transcribed to a protein and a change in cellular function. RXR is not explained, nor is TRE, TR etc. (Based on Zoeller, 2005).

Figure 4 demonstrates the current theory of TH binding to the TR. From this it can be determined where the potential sites of environmental contaminants can interfere with the TR. Contaminants may bind to the TR and activate or inhibit the action of endogenous T_3 1). This could occur by causing the interaction of TRs with various co-factors such as N-CoR or SRC-1 2). Furthermore, environmental chemicals may cause the TR to experience a different affinity for the TRE preventing the appropriate binding of the TR to TRE 3).

TH, retinoids and vitamin D directly bind their nuclear receptor and forming heterodimers with retinoic acid receptors. These receptors are already located within the nucleus and are bound to the hormone response element on the DNA (Boulton et al., 1985). In the absence of a ligand, TRs will recruit corepressors such as silencing mediator of the retinoid and thyroid hormone receptor (SMRT) and the nuclear receptor corepressor (N-CoR). Repression in this way is known as silencing. In the presence of a ligand such as TH, binding induces conformational changes causing corepressor release and coactivator recruitment (Figure 4). Coactivators such as p160/SRC (steroid receptor coactivators) induce a molecular cascade that causes gene activation resulting in transcription of DNA into protein and change in cellular function (Jugan et al., 2010). If chemicals interfere with TH action by either activating or inhibiting the action of endogenous T_3, normal gene activation could be altered. Environmental chemicals may also cause a change in affinity of TR to the TRE affecting protein translation as depicted in Figure 5 (Zoeller, 2005; Mangelsdorf et al., 1995).

1.7 Thyroid Disrupting Chemicals

Environmental chemicals may target several levels of TH-related transcriptional processes. Studies have shown that chemical contaminants found in the environment that impact the thyroid system are identified as endocrine disruptors. They interrupt the thyroid system at various points along the HPT axis. Some methods include direct interaction with TH receptors, thyroid gland stimulation, as well as, TH synthesis, metabolism and transport. Depending on the endocrine system in question, other areas within a regulatory network could also be of concern.

Change in binding of the thyroid receptor to the natural ligand can be affected by environmental contaminants that compete with the binding site of T_3 (Leatherland, 1993). If environmental contaminants compete with the binding of the TH to TR by either activating or blocking the receptor problems in growth, metabolism and reproduction could exist. Receptors such as thyroid, estrogen, androgen and several others can be targets for some environmental contaminants. Bisphenol A (BPA), a monomer of polycarbonate plastics, has shown estrogenic properties and the ability to bind estrogen receptors (Soverchia et al., 2005; Arukwe et al., 2001; Huang et al., 2010). In other studies, BPA also binds TR but not as strongly as the estrogen receptors (Yamauchi et al., 2002). These contaminants are prevalent in the environment, since they can be found in the wastewater from pulp mill effluents and industrial effluents. If a chemical has a structure similar to that of the natural ligand, there is a greater potential that it will bind the receptor and either behave like an agonist, activating a biological response as a result of binding, or antagonist which can block or dampen the biological response by blocking the receptor. This could be problematic because, if activation of the receptors as a result of chemical binding takes place, molecular responses that otherwise do not need to be activated are turned on. Contrary to this, if the compound is an inhibitor it could prevent the receptor from producing a response because it blocks the binding of the natural ligand (Figure 5) Estrogenic receptors have been well studied a wide variety of contaminants, commonly referred to as endocrine disruptors, have the ability to bind to the receptors and either activate (agonist) or block (antagonist) their activity. Chemicals such as BPA and 17-α ethinylestradiol (birth control pill) have been extensively studied due to their ability to interact with estrogen receptors (Thorpe et al., 2003; Shyu et al., 2011; Jobling et al., 1998). Some examples of example of this can be

12

seen in studies looking at reproductive alterations in fish that leads to changes in sexual differentiation (Baroiller et al., 1999), gonadal development (Gimeno et al., 1998) and reproductive disturbances (Munkittrick et al., 1998; Desbrow et al., 1998a, Kidd et al. 2007). Many studies have demonstrated reproductive effects in fish populations as a result of contaminants found in the environment (Tyler et al., 1998). Similarly, binding of environmental contaminants to the thyroid receptor could impact gene expression leading to adverse effects on organisms by altering growth, metabolism and neuronal development. Several reports have shown a wide range of chemicals such as BPA, nonyphenol (NP) and polychlorinated biphenyls (PCBs) can bind TRs and affect TH signaling (Zoeller, 2005). NP is formed by the metabolism or environmental degradation of nonylphenol ethoxylates which were widely used in industrial applications as surfactants. NP exposure was studied in adult male shubunkins (single tailed fancy goldfish) by Zaccaroni et al., (2009) using TH levels as a biomarker. The study identified that not only does NP exert an estrogenic effect, but it also disrupts TH balance. Endocrine disruptors can alter transcriptional processes at many levels (Figure 4) and numerous studies have been performed to test these mechanisms of endocrine disruption (Leusch et al., 2012). With the extensive use of chemicals globally it is imperative that experimental studies are performed to identify the associated environmental risks. If these chemicals responsible for biological responses (including endocrine disruption) can be identified then appropriate management steps are taken to manage their release and reduce their risk to the environment.
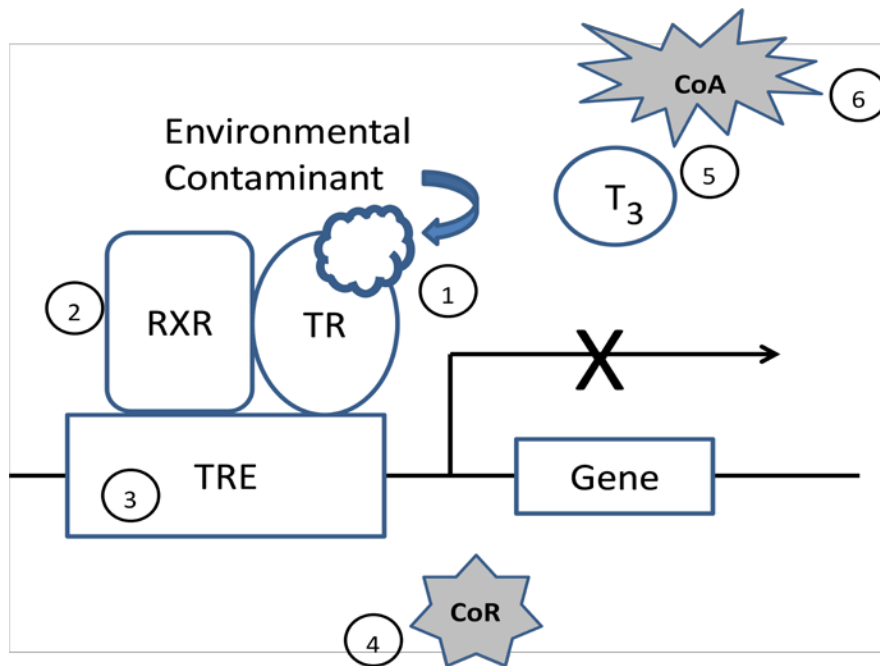
Figure 5 TH related transcriptional processes and possible target areas for environmental chemicals to impact thyroid hormone receptor regulation. 1) Expression of thyroid receptor (TR) 2) TR/retinoid X receptor (RXR) heterodimerization 3) Interaction of thyroid response element (TRE) binding with RXR and TR 4) Recruitment/release of corepressors 5) T_3 binding to TR 6) Recruitment of coactivators/transcriptional activation (based on Zoeller, 2005 and Jugan et al., 2010).

1.8 Mechanisms of Thyroid Disruption

There are different levels at which environmental contaminants interact with the thyroid system. Some include binding to the TR on target genes, others involve interruption via transport of thyroid hormone through the blood to tissues, and mechanism that either activates of deactivates the thyroid hormones. Table 1 shows examples of environmental chemicals that affect the thyroid system at these levels along the pathway.

14

1.8.1 Thyroid Receptor Binding

Regulation of transcription via the TR can be impacted in many ways along the transcriptional process. Figure 4 demonstrates all of the areas that can be impacted by endocrine disrupting chemicals. BPA was tested for endocrine disruptive properties in a study using *Xenopus* tadpoles (Yamauchi et al., 2003). Varying concentrations of BPA were used to determine the effects on the expression of TR α and β in *Xenopus* tail tissue cultures. It was determined that BPA exerted antagonistic effects on the receptor mRNA expression levels and reduced levels relative to the control in a dose dependent manner (Iwamuro et al., 2006). Figure 5, corresponds to TH related transcriptional processes and possible target areas for environmental chemicals to impact thyroid hormone receptor regulation. Areas of disruption include the expression of TR by chemicals (1). Contaminants may have the potential to affecting TR/RXR heterodimerization, however none have been identified yet (2) (Yu & Reddy, 2007). Some PCBs have been shown to impact the interaction of TRE with RXR/TR (3) (Miyazaki et al., 2004; Amano et al., 2009). The TR/retinoid X receptor heterodimer complex was partially dissociated from TRE in the presence of PCBs (Miyazaki et al., 2004). PCB's have also been shown to impact the recruitment and release of corepressors, as in a study by Iwaski et al., (2003) (4). The results from the study suggest that very low dose of PCBs have the ability to interfere with TR-mediated transactivation by impacting the TR/coactivator complex. This can eventually result in disturbed growth and development of TH target organs (Iwaski et al., 2003). Studies suggest that PCBs can also cause an affect by acting on serum T_3 and T_4 levels (Kato et al., 2004). The structures of PCBs are similar to THs and can also act as TH agonist or antagonist by binding to receptors on the TR or TTR (Chauhan et al., 2000; Kashiwagi et al., 2009). Brominated flame

retardants that have been studied for effects on TR include tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA). These studies were performed in rat pituitary cell lines that grow and secrete growth hormone in response to thyroid hormone. Both BFRs did not inhibit hormonal activity of T_3 and therefore were identified as thyroid hormone agonists (Kitamura et al., 2002). However, in subsequent studies Chinese CHO-K1 cells transfected with TRα1 or TRβ1, and TRE, demonstrated anti-thyroid hormonal action of TBBPA and TCBPA, suggesting antagonistic activity (Kitamura, 2005). Depending on the cell line TBBPA and TCBPA can act as either agonist or antagonist. More recent studies by Hwang et al., (2011) looked at methylsulfonylnitrobensoates (MSNB) as an inhibitor of steroid receptor coactivator 2 (SRC2). Analysis has suggested that MSNB inhibitors bind irreversibly to the cysteine residue (Cys 298) which is one of the four residues found within AF-2 of the TRβ. Binding causes disruption by displacing SRC2 (Hwang et al., 2011).

1.8.2 Thyroid Hormone Transport

Transport of TH through the blood to different tissues, is another area that can be affected by endocrine disruptors. Examples of these chemicals include, but are not limited to BPA, NP and diethylstilbestrol DES. DES is a synthetic form of estrogen that was previously administered during preganancy and food production. It was banned for use once it was tagged as being harmful to humans (Mattson and Batsu, 2010). These chemicals have been previously determined to interfere with the thyroid system and disrupt the organism's endocrine homeostasis (Kashiwagi et al., 2009; Murata & Yamauchi, 2008; Ishihara et al., 2003, Yamauchi et al., 2002). Binding of T_4 to TTR can be effected by flavanoids (Yamauchi et al., 2003)

halogenated PCBs (Lans et al., 1995), PBDEs (Meerts et al., 2000) and perfluorinated compounds (Weiss et al., 2009). Previous studies have suggested that several compounds found in municipal wastewater effluents (MWWEs) are able to bind to the TR (either as agonists or antagonists) and induce or inhibit downstream biochemical responses (Zoeller, 2005). A study by Yamauchi et al., (2002) looked at the binding affinities of 25 different medical, industrial and agricultural chemicals using the TR and transthyretin (TTR) assays. Both assays are used to detect competitive binding of T_3 and other test chemicals to TR or TTR (Yamauchi et al., 2002; Murata et al., 2008). They discovered that for each chemical the binding affinities were different between TR and TTR assays. Other studies have demonstrated the thyroid system disrupting activity of effluents from domestic sewage treatment plants in Japan. The results indicated that contaminants in the effluent inhibited $[^{125}I]$ T_3 binding to TR and TTR to a greater degree than the river water samples. It was also noted that there was greater sensitivity of the TR assay to the contaminants than the TTR assay. The molecular method used by Murata & Yamauchi, (2008) included three in vitro assays, TR, TTR and Luciferase (LUC), with different endpoints in order to establish the potential impact of effluent on various thyroid related mechanisms. Recombinant DNA from *Xenopus* cell lines were used in order to isolate the gene of interest and test for the specific endpoints of interest such as binding of TH to TTR and TR. The expression and purification method of recombinant proteins was carried out using cDNA encoding *X. laevis* TTR, (xTTR) and (xTR). Restriction enzymes isolated genes for both TTR and TR. Once isolated, polymerase chain reaction (PCR) amplified the cDNA. The T_3 – responsive reporter gene assay Luciferase (LUC assay) was then used to detect for binding of chemical contaminants. The basic mechanism of the LUC assay involves thyroid disruptive compounds

17

entering the cell and binding to cytosolic nuclear receptors, such as TR. The bound complex then translocates into the cell nucleus where it binds to TRE's. Normally mRNA is transcribed and protein production occurs. However, in this situation the binding of TR-ligand to TRE results in transcription of the luciferase gene which is a reporter protein and this causes bioluminescence which can be quantified using a luminometer (Schriks, 2012). More specifically this process used recombinant *X. Laevis* XL58-TRE-Luc cells created by using a permanent cell line that express a reporter gene in a T_3 dependent fashion (Sugiyama, et al. 2005a,b). A vector was introduced containing T_3 response elements (TREs) and a LUC gene downstream from the TREs to produce the XL58-TRE-Luc cells. The cells were cultured in a medium in the presence and absence of T_3 and environmental contaminants for 24 h. This assay was tested for firefly LUC activity and for cell viability in the presence or absence of environmental contaminants. When using the assay to detect impacts of sewage effluent fractions binding to the receptor they discovered that the dichloromethane/methanol fraction contained thyroid disrupting activity in TR, TTR and LUC in vitro assays however, there was no effect on cell viability at the highest concentration in all the assays. From these studies, it was evident that the LUC and TR assays were more suitable for screening of thyroid system-disrupting activity in effluents from sewage treatment plants. The LUC assay, however, was better at differentiating between agonist and antagonist activity of samples. Because of the simplicity, speed and precision the TR assay was determined to be more effective than the LUC assay (Murata & Yamauchi, 2008).

1.8.3 Iodine Transport

Iodine transport occurs in the thyroid gland via the sodium iodine symport (NIS) and this is another component of TH functioning that can potentially be impacted by endocrine disrupting chemicals. Chemicals that have been tested previously to act on these areas include perchlorates, (Wolff, 1998) chlorates, and bromates (Van Sande et al., 2003). Perchlorates, chlorates and bromates are competitive inhibitors of iodine transport into thyroid follicular cells and have been known to inhibit TH synthesis (Clewell et al., 2004). They exert their affects by competing or blocking the active inward movement of iodine across the basolateral membrane (Wolff, 1998). Studies have been conducted using FRTL-5 cells from NIS- expressing *Xenopus* oocytes as well as from non-transformed rat hepatocytes (Van sande et al., 2003; Weiss et al., 1984). The *Xenopus* cell line was used to test the transport of different chemicals such as perchlorate, chlorate and bromate. It was found that they decreased thyroidal synthesis of T_3 and T_4 by competing with or blocking the NIS transport system (Wolff, 1998; Van Sande et al., 2003). When looking at rat FRTL-5 cells results Schmutzler et al., (2006) showed that soy isoflavone genistein and 4-nonylphenol inhibited iodine accumulation. Many compounds have been identified to inhibit or block NIS, preventing TH synthesis (Table 1).

1.8.4 Thyroid Hormone Synthesis

Thyroid peroxidase (TPO) found in the thyroid gland is enzyme oxidizes tyrosyl residues in thyroglobulin to produce T_4 and limited amounts of T_3. TPO may be affected by endocrine disruptors and studies have shown that several chemicals, such as molecules known as thionamides, primarily inhibit TH synthesis by interfering with TPO mediated iodination of

tyrosine residues in thyroglobulin (Copper, 2005; Capen, 1997). Chemicals shown to have this effect are listed in Table 1. Endocrine disrupting chemicals were studied using an in vitro assay based on human recombinant TPO (hrTPO). FTC-238 thyroid carcinoma cell line transfected with hTPO were used. It was determined that BPA, bezophenone 2 along with several other chemicals inhibited hrTPO, decreasing thyroidal synthesis (Schmutzler et al., 2007**).**

1.8.5 Deiodinase Activity

Deiodinases are responsible for the activation or deactivation of THs. Inhibition and upregulation of deiodinases is another area where disruption can occur. Xenobiotics such as propylthiouracil not only inhibits TPO but was found to inhibit 5'deiodination (Cavalieri & Pitt-Rivers, 1981). Short term exposure to octyl-methoxycinnamate (UV inhibitor) also causes a decrease in serum T_3, T_4 and TSH and type I 5'-deiodinase (Klammer et al., 2007). PCBs such as Aroclor 1254 were used in studies with fetal and neonatal rats. There was an increase in whole brain type II 5'deiodinase detected in both, suggesting that in order to maintain tissue T_3 levels, type II 5'deiodinase upregulation took place to compensate for the decline in circulating brain concentrations of T_4 (Morse et al.,1993). Another study looked at the effects of erythrosine (Red dye #3), an organoiodine compound primarily used in food colouring, and determined that exposure of erythrosine in rats caused a decline in serum T_3 and an increase in rT_3, T_4, TSH and inhibited 5'deiodinase activity (Capen, 1997).

1.8.6 Sulfotransferase Activity

Sulfation is an important inactivating pathway for THs. Sulfation of the phenolic hydroxyl group blocks outer ring deiodination of T_4 to T_3. Several studies have looked at the impact of chemicals interfering with this process. In the study done by Schuur et al., (1998) the possible inhibitory effects of hydroxylated metabolites of polyhalogenated aromatic hydrocarbons (PHAHs) on iodothyronine sulfotransferase activity was tested in the cytosol of rat liver using a source of sulfotranferases enzyme in an in vitro assay with ^{125}I-labeled 3, 3'-diiodothyronine (T_2) as a substrate. It was determined that hydroxylated metabolites such as, PCBs, dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) inhibited T_2 sulfotranferase activity. The most potent inhibitor was found to be PCB metabolite (Schuur et al., 1998). Triclosan, an antibacterial agent used in household products was studied by Wang et al., (2004). Triclosan was used as a substrate and inhibitor of 3'-phosphoadenosine 5'-phosphosulfate-sulfotransferases in human liver cytosol. Increasing concentrations of triclosan were found to inhibit hepatic cystosolic sulfation of 3-hydroxybenzo(a)pyrene (3-OH-BaP) in human liver causing a decrease in sulfation of thyroid hormones (Wang et al., 2004).

1.8.7 Chemicals Responsible for Disruption

The thyroid system can be affected through a wide variety of mechanisms including those described above. Several studies (examples shown in Table 1) have been effective at targeting specific endocrine disruptors responsible for causing changes within the HPT axis. However, there are still many unknown chemicals found in effluents and the environment that may also cause disruption of the thyroid system (Subhash, 2011). The emerging approach of Effects-

Directed Assessment (EDA; described below) holds considerable promise to help isolate and

identify these biologically active chemicals in the environment (Hecker & Hollert, 2009).

Table 1 Effects and mechanisms of endocrine disruptors on thyroid hormone homeostasis (adapted from Jugan et al., 2010; Crofton, 2008; Leusch et al., 2012).

Class	Site of action	Mechanism	Effect	Compound(s)	Reference(s)
TR agonist/ antagonist	Hypothalmus/ Pituitary	1)TH receptor action to TSH mRNA 2) Deiodinase 2	Altered activation of TH gene expression	PBDE TBBPA PCB's BPA	Kojima et al. 2009 Kitamura et al., 2005 Moriyama et al., 2002
Transport Disruption	Blood	Thyroid hormone binding proteins Alter binding to serum transport proteins	1)Competitive binding with T4 binding to TTR 2) Competitive binding of T3 for thyroid hormone binding protein (THBP)	PHAH (hydroxylated PCB'S) BFR BPA Perfluorenated compounds Pentachlorophenol, pesticides, phthalates, sewage treatment effluent, DES	Lans, 1995, Meerts, 2001, Yamauchi et al., 2003, Moriyama et al., 2002; Ishihara et al., 2003
Iodine transport	Thyroid Gland	Competition or block of Na iodine symport	Decreased thyroidal synthesis of T_3 and T_4	Perchlorate, chlorate, bromated,	(Wolff, 1998; Van Sande et al., 2003)
Synthesis Inhibitor	Thyroid Gland	Inhibition of thyroperoxidase (TPO)	Decreased thyroidal synthesis of T_3 and T_4	Methimazole, propylthiourea, amitrole, mancozeb, soy, isoflavones, benzophenone 2, 1-methyl-3-propyl-imidazole-2-thione	Biegel et al., 1995, Capen, 1997, Hurley, 1998; Doerge & Sheehan, 2002; Schmutzler et al., 2007, Copper, 2005
Deiodinases	Liver HPT axis Brain and peripheral tissues	Inhibition or upregulation of deiodinases	Decreased peripheral synthesis of T_3	Propylthiouracil, PCB's, octyl-methoxycinnamate	(Morse et al., 1993; Capen, 1998; Klammer et al., 2007)
Sulfotransferases	Liver	Inhibition of sulfotransferases (SULTs)	Reduced sulfation of THs	Hydroxylated PCBs, triclosan, pentachlorophenol	Schuur et al., 1998; Wang et al., 2004; Wang & James., 2006

1.9 Effects-Directed Assessment (EDA) of Complex Mixtures

Complex mixtures, such as municipal effluents, contain many diverse chemicals that can have a variety of effects on aquatic biota. Much research is focused on the isolation and identification of these compounds so that appropriate remedial actions can be implemented to minimize damage to the ecosystem (Grote et al., 2005). An EDA allows for a rigorous approach to isolating and identifying unknown chemicals of concern in complex mixtures (Brack, 2003). EDA has become an increasingly popular technique to isolate bioactive compounds in pesticide formulations (Hewitt et al., 1998), pulp mill effluent (Hewitt et al., 2000), oil sands process waters (Thomas et al., 2009), sewage (Desbrow et al., 1998b; Snyder et al., 2001), manure (Burnison et al., 2003), and contaminated sediments (Schlenk et al., 2005).

The EDA approach helps to characterize the bioactive compounds and assists in the identification and quantification of chemicals. Each EDA procedure varies depending on the chemical composition of the mixture in question and the endpoint of interest. Extracts of mixtures (e.g. effluents) can be fractionated using a variety of physical or chemical techniques and then tested for specific biological endpoints (Grote et al., 2005). Following extraction and separation, each fraction is analyzed to determine the presence of biologically active compounds using a bioassay (Snyder et al., 2001). This will help to determine the relationship between chemicals and their effects or relevance to specific biological systems (i.e. thyroid system). The bioactive fractions can be examined for suspected contamininats or scanned for identification of unknowns, (although very difficult). The final step includes verification that the chemicals (pure standards) to see if isolated can produce the response of concern. Once a compound is found to

respond in the assay (e.g. bind to a receptor), further testing needs to be done in order to see if receptor binding affects endpoints at higher biological organization such as, system, organism, population and community. Although bioassays, such as receptor binding assays indicate the potential for a response, there are many unknown biological interactions that may occur at increasingly complex higher levels of biological organization (Routeledge et al., 1998). The bioassay response in the EDA could also be influenced by the extraction or fractionation procedures, and a change in matrix/chemical composition may alter bioavailability in the test system (Routledge et al., 1998). It is therefore difficult to predict whether a toxicant identified in the EDA will cause a response at a higher level of organization unless the mechanisms are well understood and responses are tested in whole organisms. Selection of the endpoint and bioassay is therefore critical for the predictive ability of the EDA.

In a recent study, Simon et al., (2011) used an EDA approach to isolate compounds with thyroid hormone-disrupting activity in plasma samples from polar bears. They used solid phase extraction and liquid phase extraction techniques to isolate a broad range of known and unknown thyroid disrupting chemicals. A T_4-TTR assay was then applied to measure the competitive binding activity of the various chemicals and compare them to that detected in polar bear plasma. Validations for this method required spiked cow plasma from which several Persistent Organic Pollutants (POP's) were recovered. The EDA demonstrated TTR-binding capabilities in the polar bear plasma extracts where 60-80% was a result of the presence of hydroxylated PCB's (OH-PCB's). Hydroxylated PCB's are PCB metabolites and are considered to be more potent endocrine disruptors than their parent compounds (Sandau, 2000). The measured activities in

polar bear plasma extracts were higher than the expected activities based on those calculated using the OH-PCB reference values, which suggested that there may be other unknown TH disrupting compounds present. Overall the EDA was successful in combining biological and analytical methods using chemical analysis to compounds that demonstrate biological activity (Brack, 2003).

As mentioned previously, Yamauchi et al., (2002) studied the effects of several chemicals on the binding of [^{125}I] T$_3$ to purified recombinant xTTR and to the ligand binding domain (LBD) of xTR. Results showed that there were significantly greater effects on chemical interactions with xTTR in comparison to xTR LBD. Murata & Yamauchi, (2008) examined thyroid system disrupting activity in effluents from domestic sewage treatment plants. They fractionated extracts from six different sewage treatment plants spanning agricultural, industrial and domestic sources. The TR, TTR and Luciferase assay (LUC) bioassays were all able to identify fractions of the sewage effluent possessing thyroid system-disrupting potential. Using the TTR and TR assays they were able to detect contaminants in the original water samples at 0.8 times the concentration without extraction. The assays were then applied to surface water samples in the rivers upstream and downstream of the outfall of the sewage treatment plant, from small streams in Shizuoka City, agricultural fields and effluents from industrial plants. Significant activity was detected in effluents from the sewage treatment plants and the industrial plants only (Yamauchi et al., 2003). Contaminants in the effluents were extracted using solid phase extraction (SPE) and eluted with different organic solvents however, the effluent extracts were not tested for specific chemicals. From these results they concluded that contaminants found in some wastewater effluents can

26

compete with T_3 binding to TTR and TR. It is difficult to determine if contaminants in effluents will cause an effect *in vivo* or compromise the health of the organisms when in vitro assay are being used in these experiments (Murata & Yamauchi, 2008). The potential for contaminants in Canadian wastewaters to bind to thyroid receptors from fish has not been studied and is currently unknown. From the vast amount of information uncovered through research it is evident that contaminants found in the wastewater and the environment could have an impact on the biology of organisms, potentially hindering their quality of life and survival. An EDA approach can be useful in combing the chemical and the biological data and using it to better understand the impacts associated with environmental contaminants on biological systems, thereby, bridging the gap between chemical exposure and biological effects.

Table 2 Examples of extraction and fractionation techniques used in various EDAs.

	Pulp mill effluent	Runoff	Pesticides	Sewage Effluents	Other effluents
Extraction Technique	XAD , acid partitioning with aqueous base	C_{18} Column	C_{18} SPE column	C_{18} SPE cartridges	C_{18} SPE Cartridges , aeration XAD resin coupled with DCM
Fractionation	Silica gel and/or preparative TLC	HPLC	RP- HPLC	HPLC	HPLC
Source of toxicity	Resin acids, unsaturated fatty acids, chlorinated phenolics	Equol E_2 E_1	3-trifluoromethyl-4-nitrophenol (TFM)	Estrogenicity 17β-estradiol 17β-Ethynylestradiol	Phenolics, phthalates, aliphatic carboxylic acids, aromatic carboxylic acids, amines, alkanes and linear aliphatic alcohol, 4-hydroxy-2-methylthiobenzothiazole
Reference	Marvin et al., 1995	Burnison et al., 2003	Hewitt et al., 1998	Routledge et al., 1998	Jacobs et al., 1992 ; Mazidji et al. 1990

Chapter 2

Evaluation of thyroid active contaminants in municipal wastewaters

This chapter explores the interactions of environmental contaminants with TRs isolated from fish to evaluate a proof-of-concept for application to an Effects-Directed Assessment (EDA). A competitive binding assay, based on the method of Bres et al., (1994), was developed and validated using hepatic tissue collected from rainbow trout (*Oncorhynchus mykiss*). The competitive binding assay was applied to determine if chemicals of concern that are frequently found in the environment, including BPA, nonylphenol (NP) and diethylstilbestrol (DES) were able to displace TH triiodothyronine (T_3) from trout THR. Subsequently, extracts from two municipal treatment plants, (Doon and Waterloo) were tested in the assay to determine if they contain contaminants that will interact with the TR. It was determined that BPA was the only chemical that showed binding to TR, but it bound with a very low affinity for the receptor. Because of this, it was difficult to conclude whether the issue was the sensitivity of the assay, or the binding efficacy of BPA. A stronger binding affinity was seen from the municipal treatment extracts compared to the pure chemical samples, suggesting that a combination of the chemicals could be responsible for the increased affinity. Although a proof-of-concept is presented, there remain limitations for the application of the current assay within an EDA for the isolation and identification of thyroid active chemicals in municipal wastewater.

Competitive Binding Assay

Competitive binding techniques have been used widely to determine the binding affinity of different chemical compounds to a particular receptor. Studies on estrogen, retinoid, and androgen and TRs have been conducted in this fashion (Yamauchi et al., 2002; Hewitt et al., 1998; Alsop et al., 2001; Wells & Van Der Kraak, 2000). This technique is capable of addressing questions related to receptor affinity, saturation and specificity of the receptor for the ligand. Numerous studies have compared the binding affinities of endocrine disrupting chemicals to that of the natural ligands. A competitive binding assay involves the competition of labeled and unlabeled ligand for a receptor (Boulton et al., 1985). Two important features associated with this method involve saturability and specificity, which are important in validating the technique. Saturability refers to the availability of a receptor to bind a ligand (Boulton et al., 1985). If the receptors are all bound to a ligand then they are considered saturated and thus unavailable for further binding. Specificity refers to the how precisely the ligand binds to a physiologically relevant receptor. It depends on the binding affinity of the ligand to the binding site on the receptor (Boulton et al., 1985). This means that the receptor can only bind a ligand with the specific conformation and orientation. Its specificity can be studied by evaluating how effective other similar ligands are at binding that receptor. If several unrelated ligands bind the same receptor it is considered to be non-specific. Binding affinity and capacity can be determined using Scatchard analysis. The Scatchard plot is used to linearize binding data for estimating the K_d. The specific binding curve represents the concentration of receptor binding sites and is referred to as the binding maximum (Bmax). The K_d represents the equilibrium dissociation constant such that it is a measure of the strength of interaction of a ligand to its receptor. K_d is

29

the concentration of the ligand that will occupy 50% of the receptors at equilibrium. A low K_d means the receptors have a high affinity for the ligand whereas the a high K_d means the receptors have a lower affinity (Bylund & Toews, 1993; Motulsky & Christopoulos, 2003).

Saturation binding experiments measure the binding affinity of the ligand to its receptor when at equilibrium. For example, when a chemical constituent binds to a receptor it attains a degree of attraction to that receptor. If it is not the natural ligand, it will bind with varying degrees of affinity and will produce a saturation curve that will differ from that of the natural ligand. Non-specific binding uses the same method; however, it measures the binding of the molecules that are not specific to the receptor. Therefore, total binding is the sum of non-specific and specific binding (Boulton et al., 1985). Receptor affinity can be determined by varying the concentration of the ligand in question. If low concentrations of the compound of interest displace the labeled natural ligand, then the compound has a high affinity for that receptor. This can be tested using fixed amounts of receptors and labeled natural ligand, and incubating them in the presence of increasing amount of ligand. The measure of the dissociation constant (K_d) is important in detecting the strength of binding of the ligand to the receptor. After establishing the receptor ligand affinity, a more restricted range of ligand concentration can be used for saturation analysis. When testing for non-specific binding the ligand is examined for attachment to alternative sites from the one in question (Boulton et al., 1985; Motulsky & Christopoulos, 2003). Non-specific binding (NSB) is important since the compound of interest could be binding to other non-relevant sites. NSB can be determined by saturating all the receptors of interest with unlabeled ligand and then adding radiolabeled ligand to detect other sites where the ligand may

30

bind (Motulsky & Christopoulos, 2003). Total binding (TB) is used to determine the total

number of bound sites (Figure 6). This can be done by saturating the receptors with the

radiolabeled ligand. Once this is established specific binding of the ligand can be calculated by

subtracting NSB from TB. NSB should ideally be far less than 50% of the total binding, because

this will ensure less ligand binding to non-relevant sites (Figure 6). Lower non-specific binding

will allow detection of specific binding which will be important in studying affinities of other

compounds. There should be no positive or negative binding cooperativity meaning that the

binding of one ligand to the receptor does not increase the binding affinity of subsequent ligands

to their receptors. Binding must be reversible therefore, the ligand can bind and unbind the

receptor i.e., binding is not permanent (Motulsky & Christopoulos, 2003).
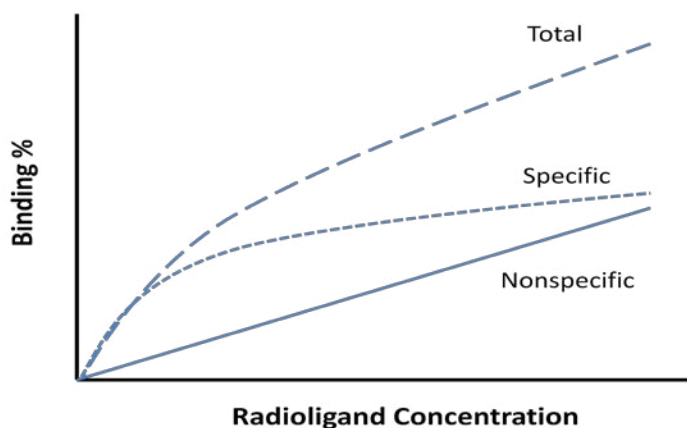
Figure 6 Example of radioligand binding data for a competitive binding assay (based on Motulsky and Neubig 2010).

Saturation binding is not enough to explain the biological relevance of the ligand/receptor

interaction. Therefore, specificity is another component that is important in competitive binding

experiments. In some instances, there can be other ligands that bind to the same receptor in which case, the receptor is considered non-specific and conformational changes may result with the binding of various ligands. A receptor that is specific for a particular ligand will not bind another ligand, since it does not possess the same conformational characteristics as its natural ligand (Boulton et al., 1985). Receptor agonists mimic the action of a naturally occurring ligand, allowing it to activate the receptor, whereas antagonists block the normal function of the receptor and in some cases depress its function (Ferkany, 1987).
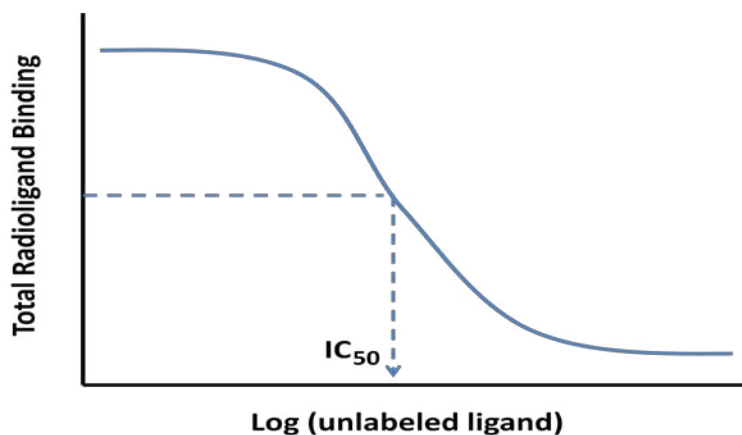
Figure 7 Competitive Binding Curve of different chemical ligands can be measured using this format (based on Motulsky & Neubig, 2010).

Overall, competitive binding experiments measure the ability of various ligands to compete with the radioligand for receptor binding sites. Competition curves are developed by creating a plot that measures the amount of radioligand bound in the presence of increasing concentration of unlabeled ligand (Figure 6). The measure of the affinity of the unlabeled ligand

32

is the concentration at which the amount of radioligand bound is reduced by 50% (IC_{50}) (Figure 7) (Fillenz, 1990).

2.1 Objectives

The objective of this work is to provide a proof of concept for applying an EDA to municipal wastewater effluents using thyroid receptor binding as the endpoint of concern. To accomplish this objective a thyroid hormone receptor binding assay using rainbow trout (*Oncorhynchus mykiss*) nuclear receptors based on the approach of Bres et al., (1994) was developed and validated using T_3. The assay was then tested using representative environmental contaminants diethylstilbestrol (DES), bisphenol A (BPA), and nonylphenol (NP). The assay was applied to extracts of final effluents from two municipal wastewater treatment plants in the Grand River watershed to determine if thyroid active chemicals could be detected.

2.2 Methods and Materials

2.2.1 Animal Preparation

Rainbow trout (Length: $26.64 \pm$ SE 2.3 cm, mass: $167 \pm$ SE 45.2 g) were obtained from Silvercreek Aquaculture in Erin Ontario. Fish were held at the University of Waterloo Wet Lab in well water at approximately 14°C and a photoperiod of 12 h dark 12 h light. The rainbow trout were fed 4P trout grower pellets (Martin Feed Mills, Tillsonburg, Ontario) every other day. After the rainbow trout were anesthetized in tricaine methanesulfonate MS 222, their spinal cords were severed and the liver was removed, being careful to separate the gall bladder. Animals were handled according to an approved University of Waterloo Animal Care protocol (AUPP 08-08).

2.2.2 Liver nuclear fraction

The liver was dissected from the fish and immediately placed on ice and washed with small volumes of Buffer 1(0.32 M Sucrose, 3 mM of $MgCl_2$, 10 mM Tris HCl, 0.1 mM PMSF, 1mM dithiothreitol, 1 mM spermidine at 7.6 pH) and Kim wipes used to absorb excess blood surrounding the liver. Liver was kept on ice until homogenized (using a Teflon glass tissue homogenizer), for approximately 5 min. The liver was homogenized using 4 volumes (w/v) of Buffer 1 per gram of liver then diluted in 4 more volumes (w/v). The homogenate was filtered through several layers of cheese cloth to remove large particles then divided into equal volumes and centrifuged at 1800 g for 10 min in order to form a crude nuclear pellet.

The supernatant was decanted and the pellet was resuspended in 30 mL of Buffer 2 (2.3 M sucrose, 3 mM $MgCl_2$, 10 mM Tris HCl, 1 mM dithiothreitol, at pH 7.6). The re-suspended

pellet solution was then layered over 5 ml of Buffer 3 (0.32 M sucrose, 3 mM $MgCl_2$, 10 mM dithiothreitol, 25 mM KCl, 2 mM EDTA, 0.5 mM spermidine, 10 mM Tris HCl, 5% glycerol, at pH 7.6). The sample was then centrifuged at 109,000 g for 45 min using a swinging bucket rotor (Beckman SW 28). The supernatant was decanted and the pellet re-suspended in 1 volume of Buffer 3. The nuclei were then separated into 1.5 mL aliquots and stored in -80°C freezer until later processing. Further processing required the sample to be thawed and the addition of one volume of a 0.25% solution Triton-X 100 (Sigma-Aldrich), in Buffer 3 and the receptor sample centrifuged at 1800 g for 10 min. The pellet was reconstituted using one volume of Buffer 4 (30 mM Tris HCl, 2 mM EDTA, 5 mM mercaptoethanol, 5 mM $MgCl_2$, 10% glycerol 0.4 M NaCl, at pH 8, 25°C) and votexed repeatedly every 5 min for 45 min then again centrifuged for 20000 g for 20 min.

After centrifugation, a pellet is formed at the bottom of the tube and the supernatant was collected. Equal volumes of Buffer 4 (without NaCl) were added to the sample to form a 0.2 M NaCl buffer solution. For example, in 1 mL of supernatant 1 mL of Buffer 4 is added to create an overall concentration of 0.2 M NaCl solution which contains the receptor.

2.2.3 Characterizing the Thyroid Hormone Receptor

Radiolabeled [^{125}I], 3,3'5- triiodo-L-thyronine - (T_3) was obtained from Perkin Elmer at a specific activity of 40.-48.8MBq/µg. Unlabeled 3,3'5 -triiodo-L-thyronine (T_3) was obtained from Sigma-Aldrich. Seven mL test tubes were prepared for total binding tests with 200 µL of receptor and 25 µL of iodinated T_3 of varying concentrations from 10×10^{-10} M to 6.25×10^{-11} M.

35

In Buffer 4, 200 µL of receptor were prepared. For non-specific binding tubes containing 200 µL of receptor, 25 µL of iodinated T_3 and 200 µL of unlabelled T_3 (at $5x10^{-5}$ M) were added. All tubes were prepared in duplicate. The tubes were covered and incubated for 24 h with gentle shaking at 4°C. After the 24 hour incubation period, 100 µL of Dextran coated charcoal suspension (Sigma –Aldrich) was added to each test tube and vortexed every 5 min for 20 min. Once the 20 min of vortexing was completed, the samples were centrifuged for 10 min at 1800 g. After centrifugation, 100 µL of the supernatant was pipetted into test tubes and counted (5 min/tube) using a Perkin-Elmer Wizard gamma counter.

2.2.4 Determination of Assay Conditions

After preparing the TR assay the concentration of labeled T_3 that provided the best binding affinity with low NSB was selected. This was done by further validating the assay, by maintaining an unchanged concentration of labeled T_3 while varying the receptor volumes. A volume of 25 µL of 0.625×10^{-10} M T_3 was added in duplicate tubes containing increasing volumes of receptor solution (50 µL, 100 µL, 150 µL and 200 µL). The concentration was adjusted using Buffer 4 to a total volume of 300 µL. Unlabelled T_3 at a concentration of 5×10^{-7} M was added to a second set of tubes to saturate the receptors in or to determine non-specific binding. Once completed the samples were incubated at 4°C for 24 h. After the incubation period, the receptor samples were treated with Dextran coated charcoal suspension and vortexed for 20 min every 5 min. Once the samples were vortexed, they were centrifuged at 1800 g for 10 min at 4°C. Being careful not to disrupt the soft pellet, 100 µL of the supernatant was placed

into new test tubes for counting in the gamma counter. The binding was described using

GraphPad Prism 5 (Motulsky, 2003)

2.2.5 Testing chemical standards and environmental contaminants

L-Thyroxine (T_4), Diethylstilbestrol (DES) a synthetic estrogen, bisphenol A (BPA), an

industrial chemical used in plastics and 4-nonylphenol (NP) a product of industrial surfactants

were obtained from Sigma Aldrich. Previous experiments have shown that above chemicals have

thyroid disruptive properties and were therefore used in the competitive binding assay.L-

thyroxine (T_4) was diluted in ethanol to a concentration of 10^{-4} M T_4, as an intermediate stock.

From this a series of dilutions from 10^{-4}M to 10^{-12}M were carried out in Buffer 4 (0.2M NaCl). In

order to avoid large concentrations of ethanol that could interfere with the assay, the dilutions

were carried out in Buffer 4.

A solution 10^{-3} M of T_4 was prepared in 97% ethanol. A serial dilution was prepared in

Buffer 4 (0.2M NaCl) solution (pH 8-9). In a test tube, 20 µL of each dilution was added to 180

µL of Buffer 4 (0.2M NaCl). In 3 separate test tubes, 20 µL of T_4 dilution and 180 µL of buffer

were added. The same process applied to T_4 was used to test the representative environmental

contaminants: DES, BPA and NP (Figure 8). The chemicals were prepared by dissolving them in

97% ethanol to achieve 10^{-4} M solution. In order to avoid damage to the sample from high

ethanol concentration, an intermediate dilution was made from the concentrated stock, for each

chemical.   From the intermediate solution 3 µL aliquots with [125] I T_3 and buffer were added to

each sample test tube. Once prepared, the samples were incubated and tested as previously

mentioned under characterization of the receptors.
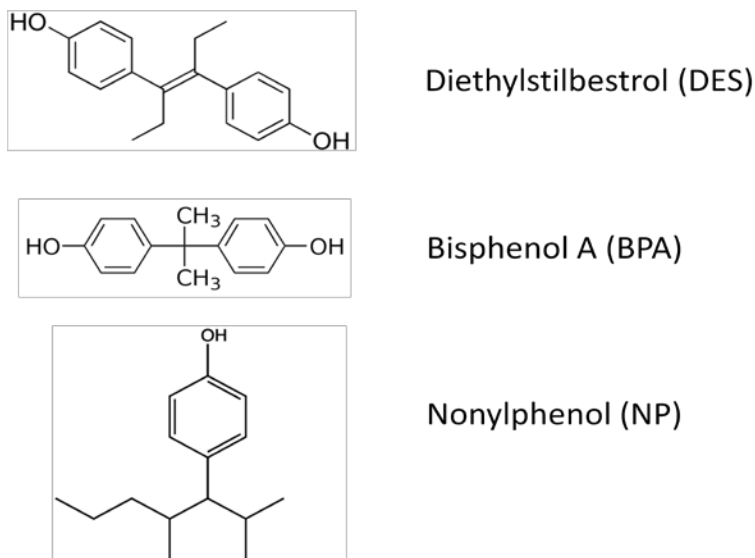
Figure 8 Structure of environmental contaminants used in the study.

2.2.6 Testing Effluent Samples

Final effluent was collected from two secondary treated municipal wastewater treatment

plants in southern Ontario: Waterloo and Kitchener (Doon) between July 2009 and August 2011.

Final effluent was collected in 1 L amber bottles, transported in coolers on ice and stored at 4°C

for less than 48 hours. Four 1 L bottles of effluent samples were filtered through 0.45 µm

Whatmann glass fibre filter paper and extracted using Oasis®HLB 60 µm, 6 cc/500 mg

cartridges. The extract was filtered through a Acrodise syringe filter (0.2 µM supor [MS1]

membrane, PN4602;) and rinsed twice with 200 µL of methanol. A subsample of the extract was

taken to dryness under a gentle flow of N_2 and reconstituted it in 100 µL of 97% ethanol. Using

the stock solution, a serial dilution was performed in ethanol at concentration ranging from 10^{-2} M to 10^{-6} M of effluent. From this, 3 µL of each serial dilution was added to sample tubes and assayed as described under characterization of receptors section.

2.3 Results

TRs were successfully isolated from the liver of rainbow trout. The isolated receptors were characterized to determine binding affinity and non-specific binding of $[^{125}I]$ T_3. A dose response relationship can be seen from the binding of $[^{125}I]T_3$ to the receptors that was distinguishable from the non-specific binding (Figure 9). Due to the difficulty of obtaining and isolating receptors, the smallest amount of receptor sample that was reliable for use in subsequent studies was determined from several tests shown in (Appendix B). It was confirmed that 36 µg were in 200 µL of receptor extract and this was sufficient to produce binding with a limited increase in NSB.

Total and Non Specific Binding [I125]T3
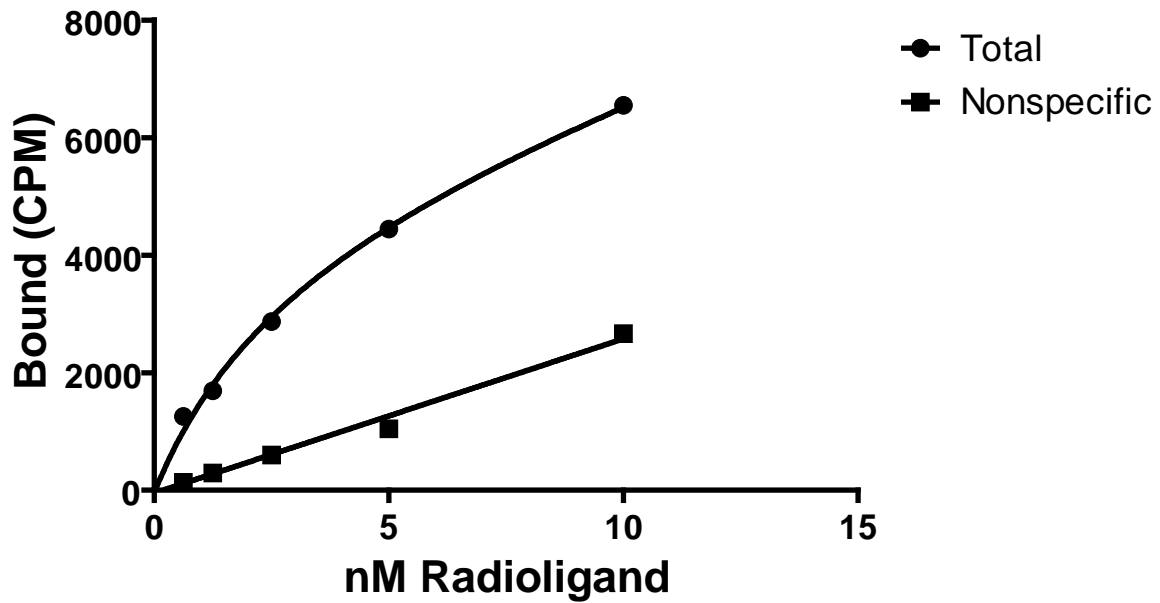
Figure 9 Introduced $[^{125}I]$ T_3 at varying concentrations to determine binding characteristics of the T_3 hormone to the THRs receptors. The R^2 values for total binding and non-specific binding were 0.9957 and 0.9866 respectively.

Specific Binding of [125I]T3
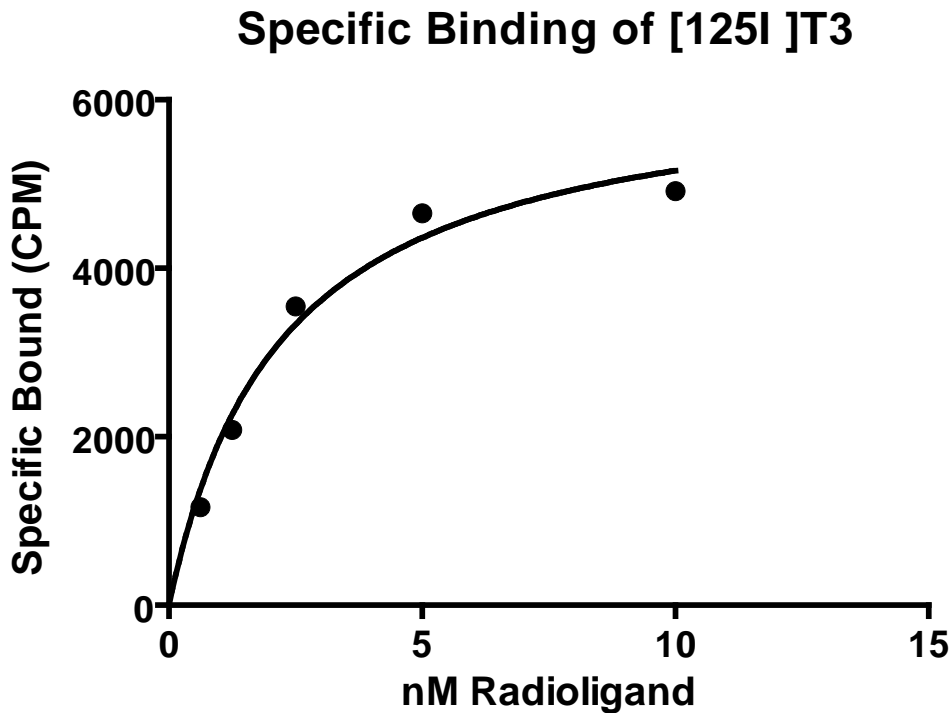
Figure 10 Specific binding curve at increasing concentrations of [^{125}I]T$_3$. Bmax and K$_d$ values can be determined with this model. The equation used to model this graph is determined from GraphPad Prism 5. The R^2 value is 0.9743**.**

The relationship depicted in Figure 10 shows the change in binding capacity of the receptors with increasing radioligand concentration. As the concentration of the labeled ligand increases there is less binding to the receptors (indicated by the gradual plateau at higher concentrations) suggesting that the receptors are approaching a point of saturation. The Bmax and Kd values for specific binding were calculated as 0.046 fmol/mg and 2.24 nM respectively (Table 3). The Scatchard analysis (Figure 11) represents the ligand/receptor binding interactions. The negative reciprocal of the slope represents the Kd value and the point at which the line

41

intersects the x axis represents Bmax. Information was used for subsequent studies with

environmental chemicals as well as effluent extracts to determine the presence of competitive

binding. Keeping NSB to a minimum was crucial in producing the best possible binding data.

NSB in this study remained the same with increasing receptor volume. At the lowest $[^{125}I]$ T_3

concentration (6.25×10^{-11}M), NSB was 10% of total binding. Specific binding however was

greater than non-specific binding, making the assay a good tool for producing competitive

binding results.

Table 3 Summary of binding affinity (Kd) and capacity (Bmax)

Hormone	Bmax (fmol/mg)	Bmax (fmol/mg) Literature*	K_d (nM)	K_d (nM) Literature*
T_3	0.046	62	2.24	0.14

*Literature values obtained from Bres and Eales. (1986)
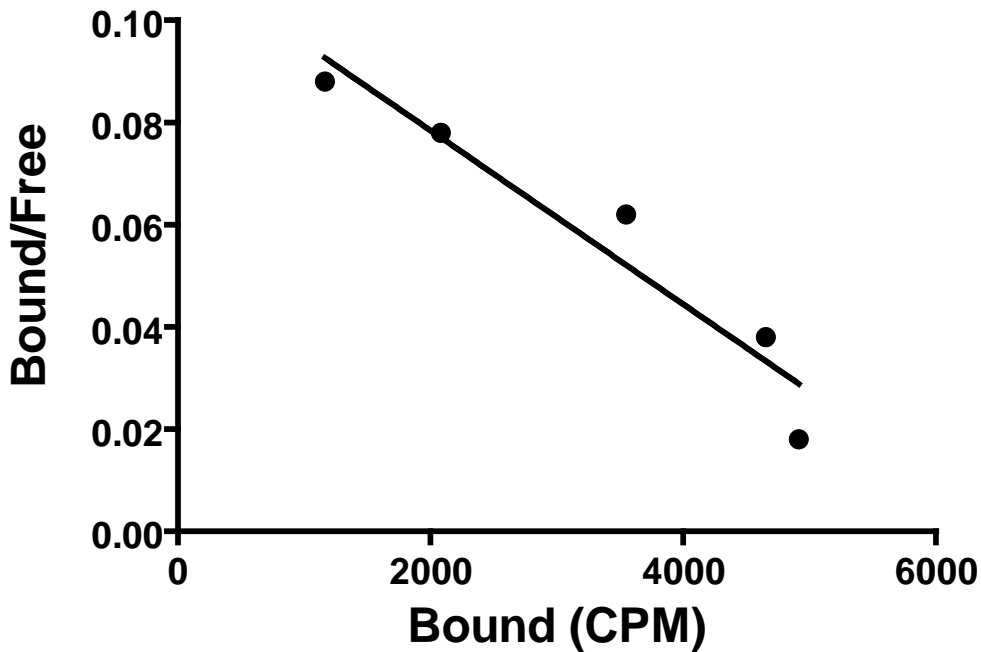
Figure 11 Representative Scatchard plot of saturable $[^{125}I]T_3$ binding to purified liver nuclear fraction. Each point represents the mean of the duplicate determinations. $R^2 = 0.9212$.

Figure 12 demonstrates the binding relationship of $[^{125}I]T_3$ to the thyroid hormone receptor at varying receptor volumes. NSB does not increase drastically and begins to form a plateau as receptor concentration increases. Therefore, the higher the concentration of the receptor used in the assay the greater the specific binding with minimal changes NSB. This test determined that the lowest concentration of receptor giving sufficient binding is $6.25 \times 10^{-11}M$. Appendix B contains additional data on the validation of this assay.

Comparision of Total, Non specific and Specific Binding
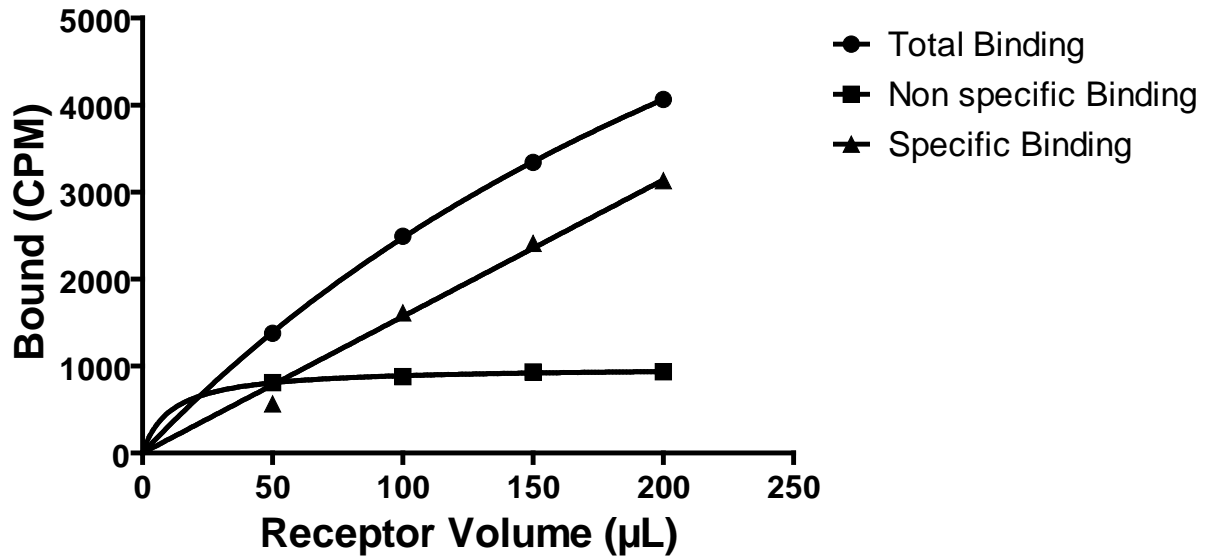
Figure 12 Nonlinear regression describing the relationship between the increase in receptor volume and the binding of $[^{125}I]T_3$. Protein concentration is .18µg/µL. Displayed here is the total, non-specific and specific binding of $[^{125}I]T_3$ at varying receptor volumes (protein concentration). Each point on the graph represents the mean of two duplicate determinations.

Competitve Binding T4
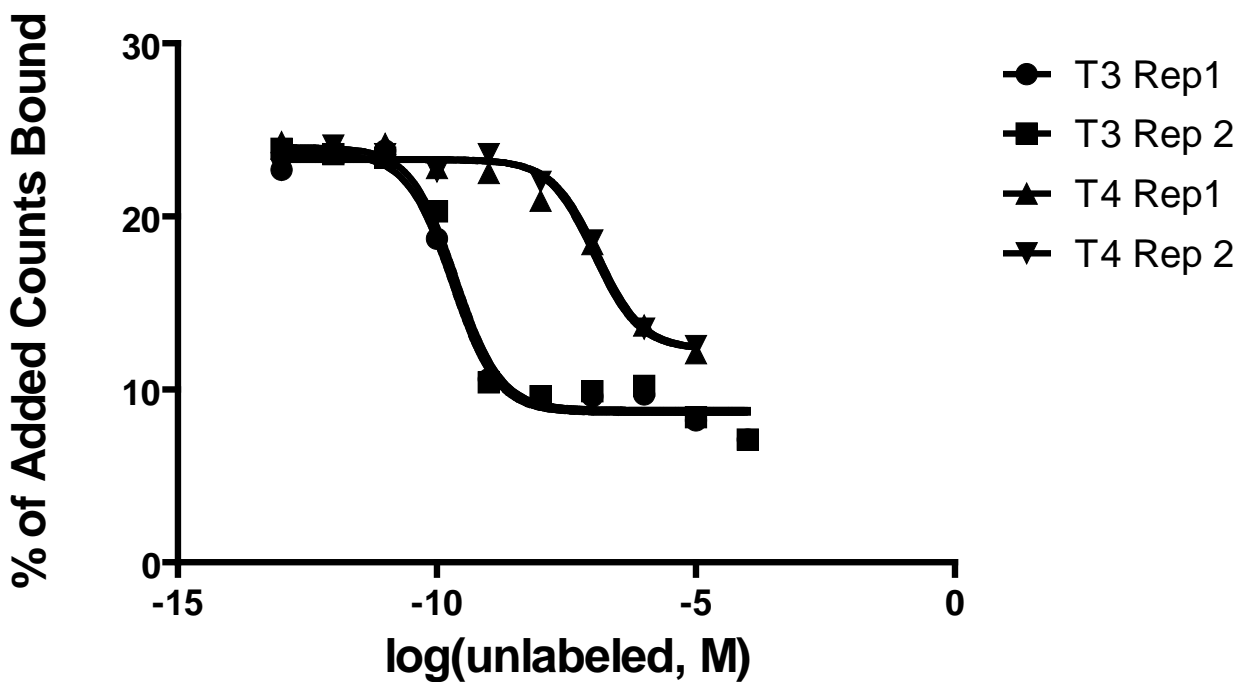
Figure 13 Competitive binding of T_4 in the presence of T_3. Each value represents the mean of two duplicate determinations.

Figure 14 indicates the competitive binding of BPA for the thyroid hormone receptor with respect to the natural ligand T_3. The equation used to model the relationship was:

$$Y=Bottom + (Top-Bottom)/(1+10^{(X-LogIC50)}) \quad \text{[Equation \#1]}$$

The top and bottom is representative of the plateaus from units of the y axis. In this experiment T_3 is used as a control to determine the relative potency of each chemical (Table 6). T_4 displays a lower affinity (1.83×10^{-3} T_4 to 1 T_3) for the thyroid hormone receptor. BPA was one of the least

potent with an IC_{50} of 6.26×10^{-5} nM and a relative affinity of 3.43×10^{-6}. Chemicals such as BPA

and NP were very insoluble and as a result, higher concentrations could not be determined. The

experiments conducted for DES and NP did not show any binding to the receptor during the first

trial and therefore further experiments were no longer continued using these samples.
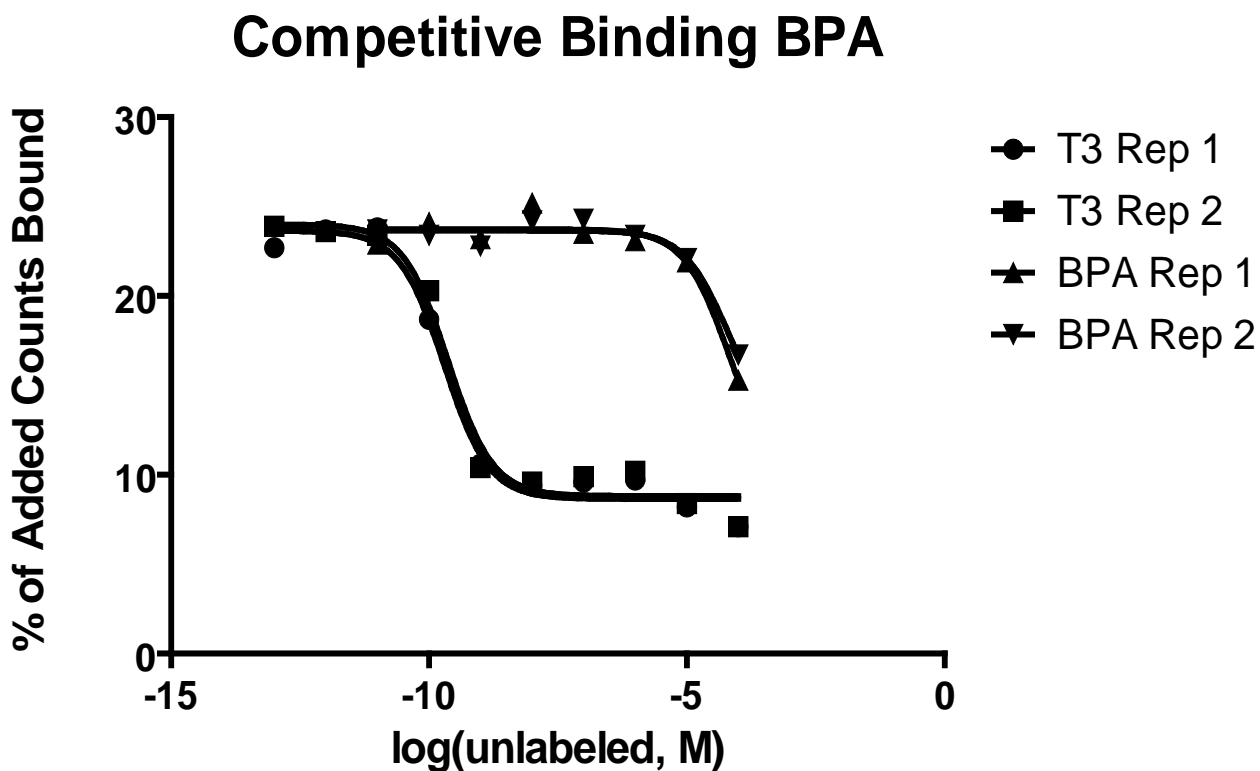
Figure 14 Competitive binding of BPA, with Thyroxine (T_4) each value represents the mean of

two duplicate determinants.

Competitve Binding Curve
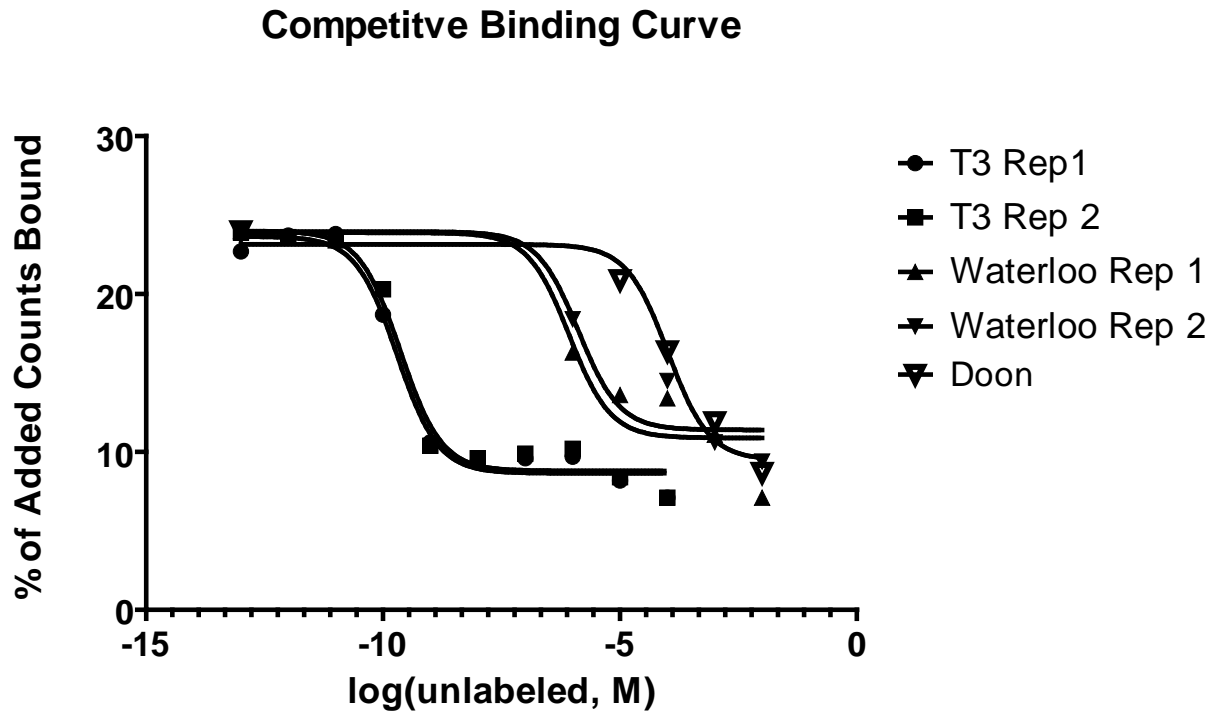
Figure 15 Competitive binding data showing the binding affinity of T_3, Waterloo and Doon wastewater effluent extracts.

Both of the wastewater effluent extracts showed considerably lower affinity relative to T_3 (Figure 15). The relative affinity of the Kitchener (Doon) extract was less than that of the Waterloo effluent extract. Table 6 shows that the relative affinity for Doon and Waterloo is 2.08 $x10^{-6}$ and 1.97 $x10^{-4}$ respectively.

Table 4 Relative affinity of chemicals or municipal wastewater effluent (MWWE) extract tested to thyroid hormone receptor.

Chemical/extract	IC_{50} (nM)	Relative Affinity (IC_{50})
T_3	2.15×10^{-10}	1
T_4	1.17×10^{-7}	1.83×10^{-3}
BPA	6.26×10^{-5}	3.43×10^{-6}
Waterloo MWWE	1.09×10^{-6}	1.97×10^{-4}
Doon MWWE	1.035×10^{-4}	2.08×10^{-6}

2.4 Discussion

In the present study a competitive binding assay using TRs isolated from rainbow trout was developed and applied. Initially, the endogenous hormones T_3 and T_4 were used in the assay to test for competitive binding. It was found that T_3 bound with a higher affinity to the receptor than its precursor T_4. This was expected as T_3 is the active form of the TH that normally binds to the receptor causing biological responses (Bres & Eales, 1986). The environmental contaminant BPA showed binding at orders of magnitude lower than T_3. The TH assay was applied to effluent extracts from the Kitchener (Doon) and Waterloo municipal wastewater treatment plants. Wastewater extracts bound to the TR with relatively the same affinity as BPA. These studies suggest that there are compounds in municipal waste waters that can bind to thyroid receptors and potentially cause biological effects in fish exposed to effluents entering the environment. The specific chemicals that are binding from these effluents remain unknown and are at concentrations higher than biological relevance.

After optimizing the assay with T_3, various environmental chemicals were tested using the competitive binding techniques. The endogenous hormone T_3, bound to the receptor with the highest affinity. This was compared to T_4, which is predominant in circulation and has 1000 times less affinity for the receptors than T_3 (Table 4). In previous papers, the binding affinity of T_4 in comparison to T_3 was 10 to 12 times lower in both rat and salmon liver samples (Darling et al., 1982). The variation in affinity could be due to different TR subtypes and isoforms. The binding affinity of T_3 to the receptor was also fairly low suggesting an overall low sensitivity of the receptor reducing the amount of bound T_4 Binding to the thyroid receptor could not be detected for either NP or DES using this assay; however another study did demonstrate binding of both NP and DES to thyroid receptors (Yamauchi et al., 2002; Kitagawa et al., 2003). In the current study BPA was capable of binding to the TRs, but only at very high concentrations (10^{-4} M). The reasons for low binding of environmental contaminants contrary to other studies could include a low sensitivity of receptor binding in the assay; or the inability to create high enough concentration of the chemical to produce detectable binding, i.e. the low solubility of NP prevented it from dissolving at high concentrations. Moriyama et al., (2002) demonstrated low affinity of BPA for the receptor as well. The affinity was approximately a hundred thousand times less when compared to T_3, which is similar to the binding affinity of BPA determined in this study. Other compounds have also been shown to bind to the TRs for example, phenols, parabens, BPA derivatives, benzenes and more (Kitagawa et al., 2003). As previously discussed, Table 1 and Appendix A, a number of studies have shown other chemicals that potentially bind to thyroid TRs. Literature Bmax values of T_3 and T_4 showed significant variations in comparison to the current study. The study performed by Bres and Eales., (1986) demonstrated a

49

higher binding capacity. The sample that was used for the purposes of the experiment did not account for changes in size, age, sex and seasonal variation of the rainbow trout. Previous studies by Bres et al., (1990) demonstrated a considerable decline in the maximum binding capacity in larger and older fish. Smaller and younger fish therefore displayed a larger maximum binding capacity. Since, the experiment did not account for changes this could be one factor that may explain the difference in the binding ability of the receptors. Although male and female fish generally express the same receptor subtypes seasonal variation does exist before or during reproductive seasons. Variation of subtypes can have a great impact on the binding capacity of the TH to the TR, because some subtypes are not able to effectively bind T_3 and others have a lower affinity. For example, TR α and TR β are both the predominant receptor substypes, within these subtypes are several isoforms which each have a different affinity for binding TH's. TRα-1 has been identified as the fully functional nuclear receptor, whereas others have limited to no effect on binding to TR (Nelson and Habibi, 2006). Fish samples were also obtained from different locations, environmental impacts could play a role in the expression of the various subtype of TR's and in those situations if less of the functional receptor is present than a lower binding capacity may occur. Proteins are extremely temperature sensitive. Some of the proteins may have been denatured causing a decline in receptors that effectively cause binding. Specific temporal changes were shown to have minimal affects on the binding capacity of the receptors however; starvation was a big factor influencing binding capacity. In the lab, the fish were not fed for approximately 3 days prior to being sampled in order to avoid feces accumulation. This may have also impacted the TR receptors binding since a decrease of maximum binding capacity was reported during starvation (Bres et al., 1990).

Wastewater effluents were tested from two large treatment facilities in the Grand River watershed. Waterloo wastewater effluent extracts showed binding affinity to the TR. Effluent extracts from the Doon (Kitchener) wastewater effluent demonstrated less binding than the Waterloo effluent. Although the binding is low it is still detectable in the assay and suggests that the wastewater effluents contain thyroid active compounds that can bind to the thyroid hormone receptors. It is known from other studies that wastewater effluent can contain a variety of chemicals capable of binding to the thyroid receptor and possessing anti-TH activity (Yamauchi et al., 2002; Sandau, 2000; Murata & Yamauchi, 2008). Both effluents used in the current study are secondary treated municipal wastewater treatment plants that do not nitrify and therefore contain high levels of ammonia and other contaminants. The effluents are likely to differ because of changes in the temporal influent composition and treatment processes. In addition, the variation in the effluent quality (matrix) may have altered the extraction efficiency and therefore composition of the final extracts (Tang & Kebarle, 1993; Taylor, 2005). Unidentified chemicals extracted from these complex effluents could have also impacted the thyroid hormone receptors in the bioassay by displacement of T_3 during the competitive binding assay. Municipal wastewater effluent contains a diversity of chemicals, from industrial and domestic sources, such as pharmaceuticals, personal care products, and pesticides (Ishihara et al., 2009) Additive or even synergistic effects may occur in these complex mixtures. The receptor binding observed in effluent extracts could be a result of single or multiple chemicals. Since the assay can only test for binding and not agonistic or antagonistic effects, other components of the effluent could also be blocking or interfering with the site of action.

There are numerous chemicals that can affect the performance of fish exposed to municipal wastewater effluents. This includes nutrients that cause euthrophication, toxic chemical such as, ammonia and alkyphenols and wide variety of contaminants of emerging concern which include endocrine disruptors (Chambers et al., 1997; Tyler et al., 1998). Fish show intersex and reproductive impairment at sites downstream of municipal effluent outfalls (i.e., Waterloo, Doon) in the Grand River (Tetreault et al., 2011). The thyroid system is very important for growth and reproduction of fish and may also be contributing to the effects observed. Ings et al., (2011) saw changes in the expression of the thyroid receptor in rainbow trout exposed to tertiary-treated municipal wastewater effluents in Guelph, Ontario, Canada (a tributary of the Grand River). Wastewater contamination in the ecosystem can cause changes and impact the aquatic environment through the continuous release of hazardous chemicals into surface waters. The detection of thyroid receptor activity in two wastewater treatment plants in the Grand River watershed suggests that there is a potential for effects on fish in the receiving environment. A number of environmental contaminants have been detected in the municipal wastewater (Metcalfe et al., 2003). The specific chemicals responsible for the effects have not been established. Several problems can exist as a result of exposure to a variety of chemicals. Polychlorinated biphenyls (PCBs) are an example of one of the many environmental contaminants that have altered the HPT axis. Exposure to PCBs has shown increased liver weight and liver lipid content in rainbow trout. An increase in the metabolism and excretion of TH and a decreased T_4 circulation can also be seen from experiments using PCBs (Brown et al., 2004). Seals exposed to a mixture of environmental PCBs displayed hypothyroidism and lower circulating levels of TH. The biological changes that correspond to exposure from environmental

contaminants are detrimental to the overall thyroid status. More specifically the primary action of

TH on target tissues is to regulate the synthesis of specific proteins through binding of T_3 to TR,

allowing for RNA transcription and translation. This action can be impeded by the presence of

chemical analogs causing disruption in the process of protein synthesis which could alter the

normal response of the organism. The biological importance of TH has been understood to have

an impact on the regulation of early fish development. Thyroid and reproductive status have

shown a positive correlation when comparing the rise in THs with gonadal maturation and

reproduction suggesting that the presence of THs can affect reproduction (Cyr and Eales, 1996).

Thyroid receptor binding effects have been studied in zebrafish on TRα. TRα is expressed

heavily in the ovary during embryogenesis during blastula and gastrula stages. This suggests that

THs and there receptors could have an impact on early development in fish. In addition to

reproduction TH also affects morphogenesis, skin pigmentation, osmoregulatory properties and

general behaviour in fish. This can be seen in parr-smolt phases of coho salmon life cycle

(Dickhoff et al., 1978). From this it is inferred that THs may have a role in maturation of

developing fish. Furthermore, change of morphology in Japanese flounder was discovered during

gametamorphosis the dorsal fin ray lengthens in the presence of exogenous TH and accelerates

metamorphosis. Alterations in the natural cycle of metamorphosis in fish could have overall

detrimental impacts to their survival (Miwa, 1985) (Appendix A, Table 5). In general, any

alteration of either development or sustenance of a species can be changed by interferences in its

natural habitat. Some alterations can result in adaptations to the surrounding while others can

cause harmful impacts that could disrupt the regular life cycle of the species. More specifically

looking at the thyroid system, there is still more work to be done on the physiological outcomes

resulting from exposure to environmental contaminants. However, from the many studies that have been performed it is apparent that TH plays a major role in growth, development and overall status of fish as well as several other species.

2.4.1 Implications

EDAs have become a popular approach to isolate and identify bioactive chemicals in complex mixtures (Hacker & Hollert, 2009). Researchers around the world have recently applied EDA using a variety of endpoints (e.g., hormone receptors, AhR, toxicity), that are mechanistically linked to environmental concerns, to isolate bioactive compounds in a wide variety of complex mixtures including pesticide formulations (Hewitt, et al. 1998), pulp mill effluent (Hewitt et al., 2000), oil sands process waters (Thomas et al., 2009), sewage (Desbrow et al., 1998), and contaminated sediments (Matthias et al., 2005; Schlenk et al., 2005). In the United Kingdom Desbrow et al., (1998) isolated natural and synthetic estrogens (EE2) from sewage effluents. These compounds had been shown to cause estrogenic effects in fish including intersex (Jobling et al., 1998). Considerable attention was later given to this approach to isolate bioactive contaminants. Using an EDA approach, a variety of hormones and the industrial contaminants including BPA, NP and ocytylphenol have been detected in Canadian sewage effluents (Burnison et al., 2002). Overall, the results in this thesis have explored the possibility of the application of an EDA approach using thyroid receptors in municipal wastewaters. The next step should be to use these techniques to isolate specific fractions (e.g. reverse-phase HPLC), to compare and contrast different effluent samples from various sources and ultimately identify the key contaminants in the effluents that have the potential to bind to the thyroid receptor. This will

help to further develop an understanding of the type of contaminants that could potentially cause an effect on TH receptor binding. With this knowledge, further studies can be performed that may help to determine the impact of contaminants on the thyroid function in aquatic species at a physiological level. More detailed and replicated studies need to be conducted on a wider range of wastewater effluents such as, from primary and tertiary treatment plants. It would also be beneficial to test effluent from other communities with different characteristic (population, treatment, etc.). In addition, there are several other assays that have been tested for TH activity such as, TTR and LUC assay. It would be beneficial to use more than one bioassay to determine the effects of endocrine disruption caused by receptor binding. Other mechanisms that are responsible for thyroid functioning within the thyroid system may be important. Binding to the thyroid receptors in the current study was only seen at very high concentrations. In contrast, other studies have seen receptor binding at very low concentrations with similar compounds such as BPA and NP (Nishiara et al., 2000). Further studies still need to be conducted in order to optimize the binding assay and to make any direct conclusions regarding the physiological effects of specific contaminants or mixtures on organisms acting through the HPT axis.

2.5 Future Considerations

The method selected in this study presented numerous challenges and limitations. Receptor preparation from liver tissue involved several homogenization and centrifugation steps in order to isolate and separate the nuclear fraction (Bres & Eales, 1986; Bylund & Toews, 1993). The purpose of carrying out multiple centrifugation steps was to eliminate any soluble interfering substance that could potentially hinder the assay, by either preventing binding of the

ligands to the receptors or by making them unavailable for binding (Bylund & Toews, 1993).

Other disadvantages associated with this method include the large sample size that was required

to achieve a sufficient amount of protein per preparation. Many rainbow trout were needed in

order to produce enough protein to run each sample, making it difficult to produce and test a

large numbers of samples.

Great care needs to be taken to ensure that the receptor was not denatured during

isolation and preparation for use in the bioassays. Sample tubes were required to be held at 4°C

and a relative pH of 7 or 8. Fluctuations in the temperature during centrifugation steps can cause

some protein denaturation, disrupting the receptor and affecting the bioassay. Ensuring that the

temperature stays within the required range is important when working with protein samples.

Avoiding multiple transfers of sample as well as, multiple freeze thaw cycle could help minimize

denaturation of the protein, making it more responsive in the assay (Boulton et al., 1986). The

assay condition could have been affected by these factors compromising the sensitivity and

specificity of the assay.

The use of $[^{125}I]$ T_3 was another limitation as iodinated compounds have short half-life

and assays must be applied within a short time frame for each batch. Iodinated compounds may

change conformation and alter the binding affinity causing discrepancies when validating the

assay (Motulsky & Christopoulos, 2003). Advantages to iodinated compounds are that they have

a higher specificity, making them beneficial when receptor density is low or there is less tissue

(Bylund & Toews, 1993). However, iodinated compounds are more difficult and hazardous to handle and work with.

The binding resin (Dowex) that was used in the paper by Bres et al., (1986) produced very high NSB in this study. After numerous experiments, the resin was eventually isolated as a possible cause of the excessive NSB. Once the Dowex resin was replaced with Dextran charcoal suspension the NSB decreased significantly with the increased concentration of the labeled ligand.

When analyzing the data several assumptions have to be made, such as the affinity of the labeled and unlabelled ligands is identical, but it is possible that there may be some differences in the affinity due to the radioactivity of the radioligand (^{125}I). Iodinated compounds can change the conformation and binding affinity of the ligand (Motulsky & Christopoulos, 2003). Another assumption is that cooperativity does not occur, meaning that the binding affinity of one site does not increase with the binding of another. It is assumed that there is no ligand depletion, suggesting that free concentration of radioactive and non-radioactive ligand equals the added concentration. Lastly, a proportion of radioactive ligand binds non-specifically, despite the concentration of the unlabelled ligand present (Motulsky & Christopoulos, 2003). The labeled ligand also binds to other proteins besides the receptors that are located in each sample (referred to as NSB) and can interfere with the performance of the assay. The assumptions made are important because if they do not hold they will affect the interpretations of the results.

Overall, the competitive binding assay method applied, similar to that of Bres et al., (1994), was challenging, yet effective and once the procedure was optimized, the assay was simple to apply. The thyroid hormone receptor extraction method had several advantages once it was functioning optimally. Such as, running multiple experiments simultaneously was simple and quick and preparing the samples for incubation was done fairly rapidly allowing large amounts of data to be collected at one time. Although the assay was relatively simple and quick, the method was not very sensitive, limiting its potential application in future studies. Despite successfully demonstrating the application of this technique, alternative methods that are more sensitive should be considered alongside the TR binding assay in the future. Use of TTR and LUC assay can help provide thorough results pertaining to binding characteristics of contaminants to TR. In addition, questions regarding the binding capabilities of certain chemical compounds as well as effluents can be confidently answered with the use of multiple or more sensitive assays. This assay would not be a viable technique for a risk assessment because the method was not effective at detecting chemicals at environmentally relevant concentrations. It could be used as a screen to provide an indication of binding however an accurate determination of risk would require validation of effects at higher levels of organization.

Appendix A

Table 5 below represents a list of environmental contaminants that impact thyroid system functioning. Several studies have been performed to determine the site of action of the following chemicals and in what ways they are capable of impacting the thyroid system. These are a few of the many environmental contaminants that have been studied that impair thyroid system functioning in various organisms.

Table 5 Different classes of chemicals associated with thyroid hormone disruption.

Environmental Contaminants	Source	Mechanism	Effects	Chemicals	References
Polychlorinated Biphenyl Mixtures	Dielectric fluid, Hydraulic fluid, Printing inks, adhesives and paint	Mode of action not well understood	-Lowered plasma T_3 levels of salmon -smolting coho salmon→ increased plasma T_3 delayed onset of Na^+-K^+ATPase	_____	(Leatherland & Sonstegard, 1978) (Leatherland & Sonstegard, 1980)
Planar halogenated aromatic hydrocarbons (PHAH)	By products of chlorinated phenols Chlorinated phenol derived products Commercial PCBs-Aroclor, Clophen, Kanechlor Arise from diverse combustion sources	Bind to aryl hydrocarbon (ah) receptor and induce cytochrome 1A gene transcription (CYP1A)	European Flounder-depressed plasma total T_4 (TT_4)levels and -inhibits metamorphosis juvenile rainbow trout- PCB treatments lowered T_4 and T_3 levels in muscle	2,3,7,8-TCDD 2,3,4,7,8-PCDF 3,3',4,4'-tetrachlorobiphenyl 3,3'4,4',5-pentachlorobiphenyl Naphthalene Phenanthrene Pyrene 3-methylcolanthrene	(Besselink et al. 1997) (Schreiber & Specker, 1998) (Brown, et al., 2002)

Organochlorine Pesticides	Mirex- Fire retardants in plastics Paint Electrical goods Endrin- wood preservatives and insecticide/ acaricide on food crops	Endrin – interferes with GABA receptors	Rainbow trout or coho salmon (mirex)- both reduced T_3 T_4 levels and altered thyroid histological appearance Endrin- blocks TH synthesis by direct action on thyroid or by compromising pituitary TSH production and release	Mirex Endrin DDT Endosulfan Lindane	(Leatherland & Sonstegard, 1979) (Shukla & Pandey, 1986) (Pandey et al. 1995)
Environmental Steroids	Sewage effluents and runoff from livestock operations	Effects TH receptors in target tissues	E2 enhances thyroid function in Indian teleost and suppresses function in other species in immature rainbow trout E2 caused depressed plasma T_4 and altered T_4 plasma kinetics causing decreased T_4 conversion to T_3 Estrogenic compounds induce hypothyroid condition in fish	Estrogens Progesterone Androgens and their metabolites Alkyphenols 4-nonylphenol	(Sage & Bromage, 1970) (Cyr et al. 1988) (Flett & Leatherland, 1989)
Brominated Flame Retardents	Building materials, synthetic textiles, plastic products, electronic equipment	Thyroid gland function and regulation, Thyroid hormone metabolism, Thyroid hormone transport mechanism	Potent binding competitors	Tetrabromobisphenol A Tetrachlorobisphenol A	(Kitamura, et al. 2002)

Validating the Assay

Binding of thyroid hormone at increasing receptor volumes

The thyroid hormone receptor tested to determine the lowest possible volume that could be used to produce optimal results for this assay. It was determined that 200 µL would be most acceptable volume to optimize the assay and use of isolated receptors. The protocol described by Bres and Eales, (1986) used 400 µL.

T_3 Receptor Binding
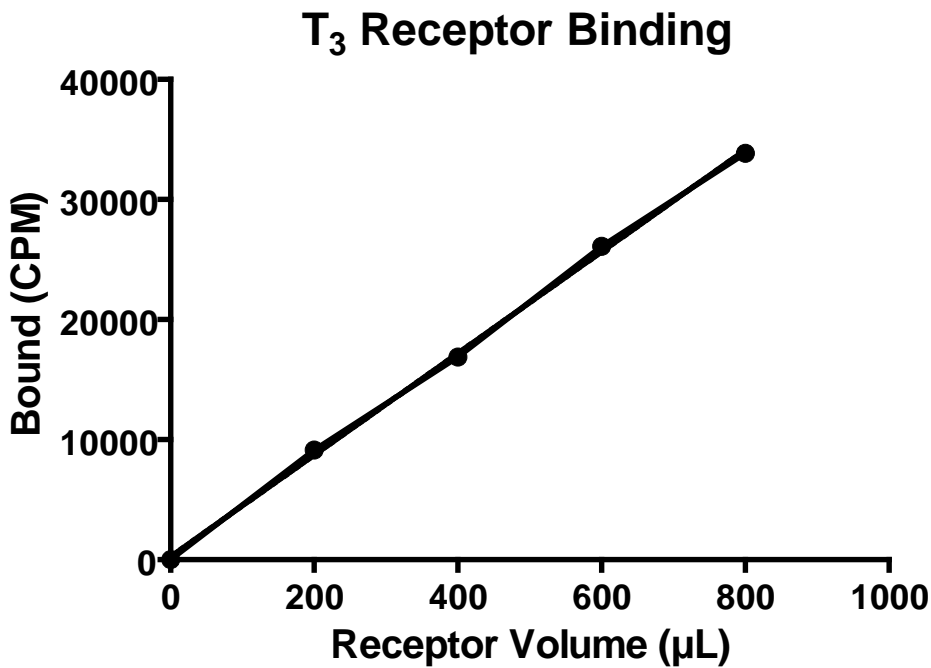
Figure 16 Relationship between receptor volume and receptor binding. At these volumes the non-specific binding remains relatively constant. The $R^2 = 0.9991$.

2.5.1 Protein analysis

A protein analysis of the receptor sample was performed to determine the concentration of the protein in the receptor extract. This was carried out to verify that indeed there was a sufficient protein content in order for binding studies to be conducted. This shows a linear relationship between protein concentration and the absorbance. Figure 17 represents protein absorbance curve the receptor samples were tested against the standards and were found to fit on the line. Triplicate sample of receptor were used without a dilution. The three receptor samples are indicated by this shape Δ.

Protein Standard Curve

$y = 2.911x + 0.309$

$R^2 = 0.988$
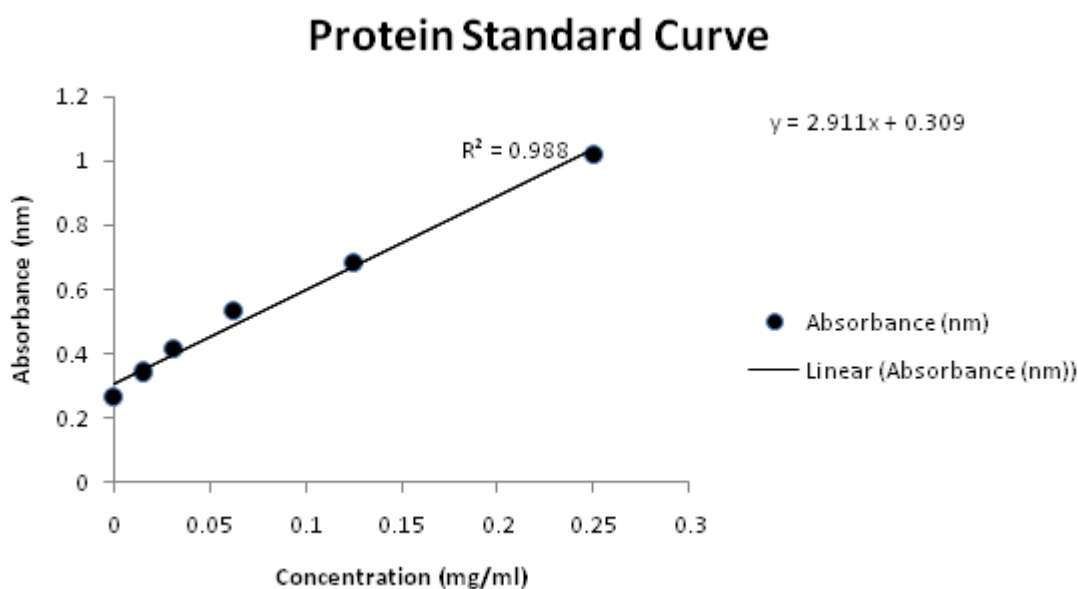
Figure 17 Bovine serum albumin was used to create a standard curve. Based on the standard curve the concentration of the protein in the liver nuclear fraction was .18µg/µL this was established using the Bicinchoninic acid method. The R^2 =0.9921 and the equation for this line is y= 2.911x + 0.309. Based on the standard curve the concentration of the protein the liver nuclear fraction is .18µg/µL using the Bicinchoninic acid method.

2.5.2 The counts per concentration of [^{125}I] T$_3$

There is a linear relationship between total count and concentration of the radioligand. The linear relationship was determined to validate, that with increasing concentration of ^{125}I there was also an increase in CPM, ensuring that the radioactive compound was good for use in further experiments. The radioactive iodine was applied to evaluate the percent binding against counts added. Each point represents the average of triplicate determinations.

Total Count Tubes [I125]T3
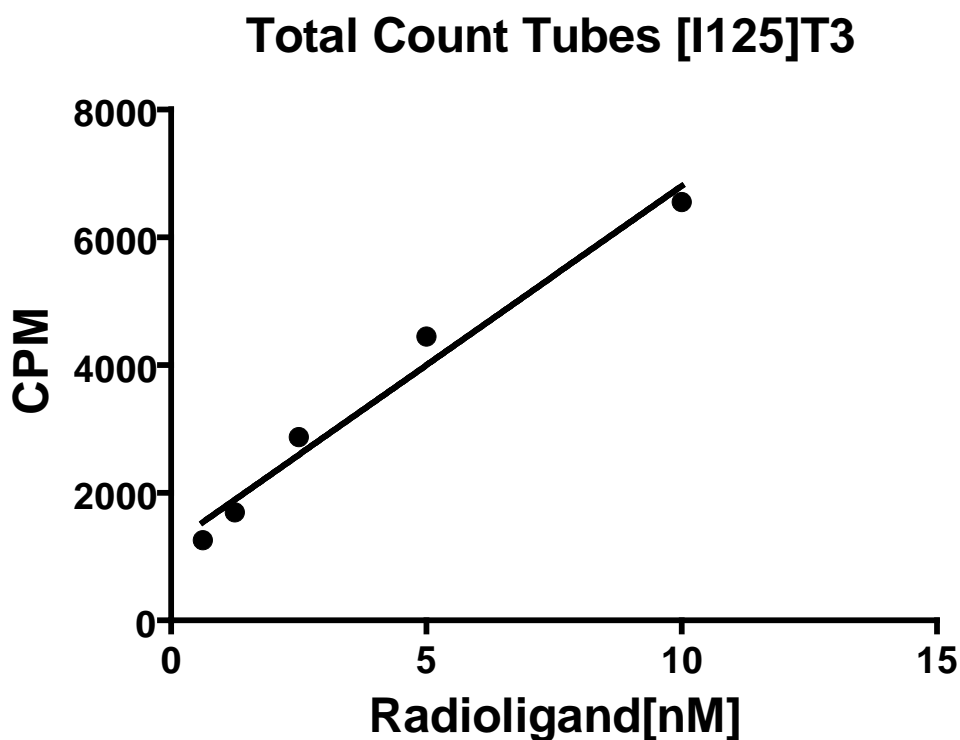
Figure 18 Determination of the total amount of radioactivity present at each concentration of [^{125}I] T$_3$. Total count tubes containing [^{125}I] T$_3$ at concentrations ranging from 0.625 nM to 10 nM. $R^2 = 0.9754$ and the equation for the line is y=561x+1186.

Lactate Dehydrogenase Assay (LDH)

Lactate dehydrogenase (LDH) is a soluble enzyme normally found in the cytosol of cells.

 Damage or lysis to the cell causes a loss of membrane integrity, when this occurs; LDH enzyme appears in the nuclear fraction as a means of showing that the preparation is contaminated. The lactate dehydrogenase assay was used to determine if the nuclear fraction was contaminated with cytosol. The results indicate that there was little cytosolic contamination with the receptor sample during intermediate and final steps of the purification procedure of the receptor extract. The assay was performed exactly as described previously (Ings et al., 2012).

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