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Assessment of androgenic response potential of effluents using in vitro and in vivo methods

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ASSESSMENT OF ANDROGENIC RESPONSE POTENTIAL OF EFFLUENTS USING *IN VITRO* AND *IN VIVO* METHODS

by

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B.Sc. Ryerson University, 2000

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Abstract

ASSESSMENT OF ANDROGENIC RESPONSE POTENTIAL OF EFFLUENTS USING *IN VITRO* AND *IN VIVO* METHODS

Emil Bandelj, M.A.Sc. Thesis, 2004
Department of Environmental Applied Science and Management
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The androgenic potential of a New Zealand pulp mill effluent (PME) and a Canadian PME was assessed along with a New Zealand sewage treatment plant effluent (STP) using a combination of *in vivo* and *in vitro* methods. The *in vitro* methods included: 1) a fish-based androgen receptor binding assay, 2) a fish-based aromatase inhibition activity assay, and 3) an analysis of gonadal sex steroid levels in exposed female mosquitofish (*Gambusia affinis*) ovaries by radioimmunoassay. The *in vivo* method included a quantifiable analysis of anal fin ray length for female mosquitofish exposed to the effluents.

Effluent extracts for the Canadian PME and New Zealand STP were found to have low *in vitro* androgenic potential compared to upstream reference extracts. All effluent extracts (Canadian PME, New Zealand PME and STP) showed a low degree of *in vitro* aromatase inhibition potential compared to upstream reference extracts. *In vivo* analysis showed no androgenic potential of the New Zealand PME and STP. The *in vitro* androgen receptor assay and *in vivo* mosquitofish bioassay did show androgenic responses for androstenedione (AD) and 1,4-androsta-diene-3,17-dione (ADD), which are two products of the microbial conversion of β-sitosterol (a plant sterol commonly found in PME) by *Mycobacterium smegmatis*. Also, the potential of the mosquitofish bioassay to determine anti-androgenic effects in effluents was demonstrated.

Acknowledgements

As is common with these types of endeavours, there are multitudes of people to thank. First and foremost, my supervisors, Mike van den Heuvel and Lynda McCarthy. Mike was always available for my queries *in situ* while I was in New Zealand, and *ex situ* while I was back home in Canada. His guidance, breadth of knowledge, and enthusiasm (particularly when mine started to silently wane) were crucial to me. Lynda's expertise and guidance were monumental to me in the writing process. My gratitude to both of them for being such an excellent pair of supervisors.

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A plethora of other people to thank down in New Zealand as well. Megan Finley contributed more than a lot of help with the effluent collections and mosquitofish bioassays. Kim McGrouther, who had to put up with me as her flatmate for two tours of duty. Murray Smith, another part of the beer-drinking and BBQ-ing Canadian contingent. Sonia Foote, Rachel Fahy, Rosanne Ellis, Sheree Anderson, Kate Wingate, Murray Robinson and anyone else I've failed to remember who helped in any part. To all the other kiwi and international friends I've managed to make, upset and still keep, including Alastair Stewart, Tobi Stegk, the Forest Research soccer and volleyball teams, and still others from France, Sweden, Germany and the UK. My experience "down under" has been wonderfully memorable.

A tremendous thanks to my family, who had to put up with all the stress, mess, anguish and periods of space-outs I subjected them to over the course of this work. And finally, to Judith, who experienced some of the ups and downs all the way across the Atlantic. It's finally over now, babe – let's get on with it!

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1.0 INTRODUCTION

There have been increasing global concerns over the past two decades regarding the exposure of wildlife and humans to chemicals that have the potential to interfere with the endocrine system. Rachel Carson (1962) first voiced these concerns, and since then there has been considerable research conducted to justify them. Knowledge of the endocrine system has increased vastly, only to show greater complexities and intricacies that further the need for research. Much study has also been devoted to isolating and identifying chemicals causing adverse effects. Studies involving fish have been successful in that they have been able to demonstrate adverse effects upon exposure to chemicals in effluents (Sumpter, 1995; Van Der Kraak *et al.*, 1992). Understanding the mechanisms of endocrine-disrupting compounds and identifying their effects continue to be major challenges. Therefore, any study investigating endocrine disruption must first begin with a review of the endocrine system.

1.1 The endocrine system

The endocrine system involves complex interactions between the nervous system, organs and chemical messengers known as hormones. It is a well conserved system within the vertebrate group with several hormones, such as the sex steroids testosterone and 17-β-estradiol, being common among all species (International Programme on Chemical Safety (IPCS), 2002). The endocrine system is responsible for regulating energy metabolism, growth, and reproduction. The intricate chemical messaging system and "cross-talk" between endocrine system pathways leads to dynamic control of release, suppression, and synthesis

of hormones within the organism (IPCS, 2002). A major class of hormones that are responsible for sexual activity and development are known as sex steroids. Sex steroids are lipid-soluble and their main route of travel within the organism is via the circulatory system (Lehninger *et al.*, 1993). They are usually bound to a protein or globulin in the bloodstream with a small percentage being un-bound or free. This balance supports the quick release of the steroid when appropriate internal and external cues are signaled and also allows for attenuation of its synthesis with the steroid usually acting as the agent in a negative feedback loop (IPCS, 2002).

The main axis of organs involved in the maintenance of sexual function within vertebrate organisms is the hypothalamus-pituitary-gonadal (HPG) axis (Starr and Taggart, 1995). Combinations of external (physico/chemical) and internal (chemical) cues stimulate the hypothalamus in the brain, which in turn begins the series of chemical messaging within the HPG axis. The hypothalamus releases gonadotropin-releasing hormones (GnRH), which stimulate the anterior pituitary to release gonadtropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) in mammals, and gonadotropin hormone I (GTH-I) and GTH-II in non-mammals (IPCS, 2002). The gonadotropins travel through the bloodstream and make their way to the gonads, where they stimulate the production of androgens and estrogens, both of which play roles in masculine and feminine development respectively. Figure 1.1 shows a flowchart representation of the HPG axis discussed.

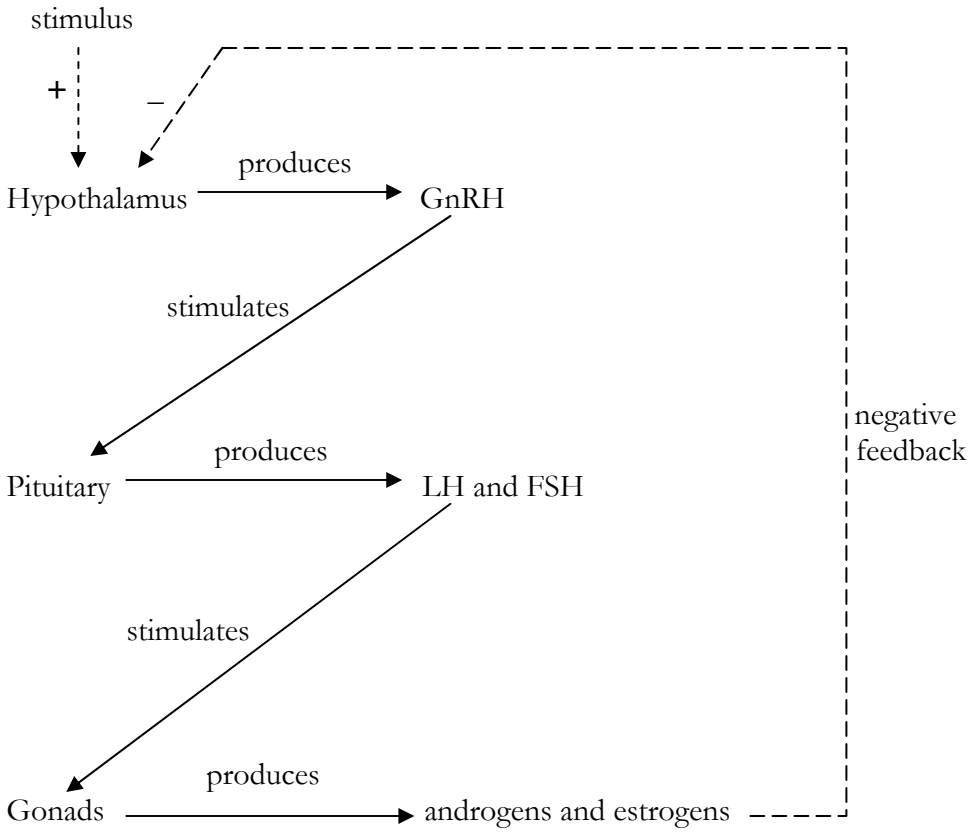

Figure 1.1: Flowchart representation of hypothalamus-pituitary-gonadal axis in vertebrates

Secreted sex steroid hormones, synthesized and released from gonadal cells, find their way to target cells by diffusion or by transport via sex steroid binding proteins (SSBP) (Lehninger *et al.*, 1993). The steroid enters the target cell and diffuses to the perinuclear region where it binds to its specific receptors. The receptor-ligand complex then combines with transcriptional factors that allow it to bind to specific DNA sequences coding for other proteins including hormones, enzymes, growth factors, or cellular structural components (Starr and Taggart, 1995). The binding of the receptor-ligand-transcriptional factor complex, known as a hormone response element (HRE), initiates mRNA synthesis (IPCS, 2002). The mRNA diffuses out from the perinuclear region and

undergoes further translation into proteins by ribosomes on the endoplasmic reticulum in the cytosol. Figure 1.2 below diagrammatically shows the general mechanism of steroid hormone action.

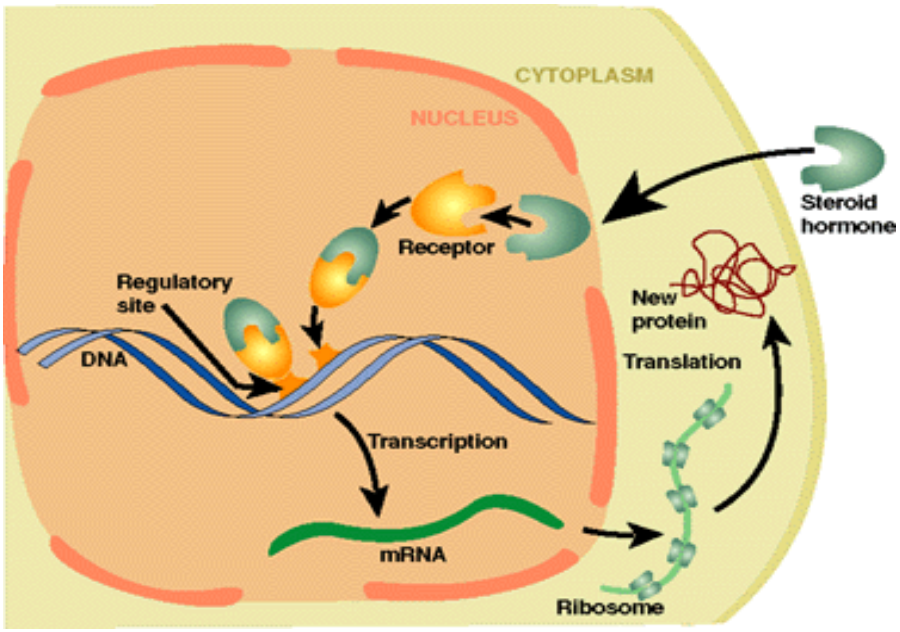

Figure 1.2: Mechanism of steroid hormone action on target cell (from http://e.hormone.tulane.edu/learning/targetcells.html)

1.2 Androgen receptor binding

Development of male sexual characteristics in vertebrate organisms is dependent on the binding of androgens to their specific receptors (Lehninger *et al.*, 1993). Once bound to its receptor, the steroid-receptor complex goes on to elicit a response as according to the mechanism shown in Figure 1.2. Since steroids and their receptors generally have a very high and unique affinity for each other, the respective protein products from their interaction are also unique. For example, the interaction of the estrogen 17-β-estradiol with the estrogen receptor in fish elicits the production of the egg yolk protein vitellogenin (VTG). This has

been shown to happen in male fish exposed to estrogens in the wild (Larsson *et al.*, 1999; Sumpter, 1995; Jobling *et al.*, 1998). Under natural conditions, only mature female fish produce high levels of VTG, thus VTG induction in wild species of male fish is a highly selective indicator of environmental estrogenic contamination (Sumpter and Jobling, 1995). Recently, androgenic responses measured by the production of the male glue protein, spiggin, in female sticklebacks have been shown to be achievable through water-borne exposures to the androgen dihydrotestosterone (Katsiadaki *et al.*, 2002). A six-week exposure of female sticklebacks to pulp and paper mill effluent (PME) also showed significant induction of spiggin (Katsiadaki *et al.*, 2002), but no other studies measuring spiggin induction are available yet.

Steroid receptor binding assays have also been used for demonstrating androgenic (Wells and Van Der Kraak, 2000) and estrogenic (Thomas and Smith, 1993) responses in fish exposed to xenobiotics. Receptor binding assays exploit the fact that steroids have a high and unique affinity for their respective receptors (Lehninger *et al.*, 1993). Thus, demonstrating the binding potential of a xenobiotic to a specific receptor may possibly be used to infer a potential response in the organism. The fish androgen receptor was first characterized in the skin of male brown trout (Pottinger, 1987), and subsequently in goldfish brain (Pasmanik and Callard, 1988), and rainbow trout lymphocytes (Slater *et al.*, 1995). The characterized fish androgen receptor was found to have a high affinity for testosterone, along with a low affinity for an important fish androgen, 11-ketotestosterone. Different physiological roles have been deduced for testosterone and 11-ketotestosterone in fish. Testosterone has been shown to

control gonadotropin secretion in both male and female rainbow trout by feedback (Breton and Samroni, 1996), while 11-ketotestosterone is believed to control gonadal differentiation (Piferrer *et al.*, 1993) and sexual dimorphism (Brantely *et al.*, 1993) in male chinook salmon. Subsequently, two different isoforms of the androgen receptor have been isolated and characterized from Atlantic croaker brain and ovary tissue (Sperry and Thomas, 1999). The ovary androgen receptor was found to have a higher affinity for 11-ketotestosterone than the brain androgen receptor. The physiological role of androgen receptors in females is currently unknown. In theory, therefore, the binding of a xenobiotic to the androgen receptor in fish may elicit a masculine physiological response.

Endocrine-mediated responses through the binding of androgen receptors are commonly studied *in vitro* through the utilization of competitive binding experiments involving an androgen receptor (AR) preparation, which consists of androgen receptor protein normally isolated from brain or gonadal tissue. The androgen receptor is then exposed to a pair of competitors: a radiolabelled steroid hormone and an unlabelled steroid (or xenobiotic). By increasing the unlabelled steroid concentration, its ability to displace the radiolabelled steroid can be quantified and compared with other compounds. Androgen receptor binding potential of DDT metabolites have been quantified to show limited binding (Wells and Van Der Kraak, 2000) using androgen receptor isolated from goldfish testes. The same assay has also been used to find significant binding of liver extracts from male white suckers exposed to PME (Hewitt *et al.*, 2000).

Compounds that bind to the androgen receptor but do not go on to elicit a response are known as anti-androgens, although there are other mechanisms

through which they may operate. Androgenic compounds are thus known as "agonists" since they elicit physiological responses, and anti-androgenic compounds are known as "antagonists" as they merely block the potential binding of an androgen to its receptor. An anti-androgen reduces the manifestation of masculine responses but does not induce estrogenic responses. Metabolites of the fungicide vinclozolin have been shown to produce anti-androgenic effects in male rats by inhibiting growth of androgen-dependent tissues while showing no affinity for the estrogen receptor (Kelce *et al.*, 1994). The mammalian anti-androgen cyproterone acetate has been found to be non-binding to rainbow trout brain androgen receptor (Wells and Van Der Kraak, 2000) and Atlantic croaker brain androgen receptor (Sperry and Thomas, 2000). Studies of anti-androgenic effects in fish exposed to effluents are not available.

1.3 Role of aromatase

Aromatase is an enzyme of the P450 class of steroidogenesis pathway enzymes responsible for the conversion of androgens to estrogens, the most common reaction being testosterone to 17-β-estradiol (Lephart *et al.*, 2001). It is near the end of the steroidogenesis pathway shown in Figure 1.3. The aromatization of androgens to estrogens occurs in the endoplasmic reticulum and is classified as a mixed-function oxidase reaction (Lephart *et al.*, 2001). As well as converting testosterone to 17-β-estradiol, aromatase also converts androstenedione to estrone (Figure 1.3), which is a less potent estrogen (Lephart *et al.*, 2001). The aromatization reaction requires NADPH as a reducing agent and O_2 for the three sequential hydroxylations that occur for each estrogen

molecule formed (Lephart *et al.*, 2001). Aromatase can be found in a wide variety of tissue, but it is believed to be most important at the sites of major steroidogenesis such as the gonads and brain (Antonopoulu *et al.*, 1999) such that it can control GnRH production (see Figure 1.1).

Thus, aromatase plays a crucial role in the maintenance of the androgen/estrogen ratio. Aromatase inhibition may skew the proportion of androgens to estrogens (Séralini and Moslemi, 2001). Inhibition of aromatase activity has been shown to have effects *in vivo* in male rats by causing aggressive behaviour (Turner *et al.*, 2000). This is believed to be due to the increase in the androgen/estrogen ratio due to the blockage of androgen conversion to estrogen. In fish, aromatase inhibition induced earlier spermiation in adult coho salmon males compared to control groups (Piferrer *et al.*, 1994), which is again attributed to the increase in the androgen/estrogen ratio. The androgen/estrogen ratio is important in females for inducing ovulation or apoptosis of oocytes (Séralini and Moslemi, 2001). Aromatase inhibition in genetically female chinook salmon induced development of phenotypic males when administered at a critically-timed developmental stage (Piferrer *et al.*, 1994). Thus, aromatase inhibition has the potential to show androgenic or masculine effects in fish.

Fungicides and other pesticides have been shown to inhibit aromatase *in vitro* (Andersen *et al.*, 2002). *In vivo* effects believed to be seen in rats include a marked decrease in steroid production, as evidenced by a decrease in sexual activity in male rats exposed to the fungicide fenarimol (Gray *et al.*, 1998). Since these male rats were exposed daily from weaning to adulthood, it was not unexpected that aromatase inhibition of such duration and dose would lead to

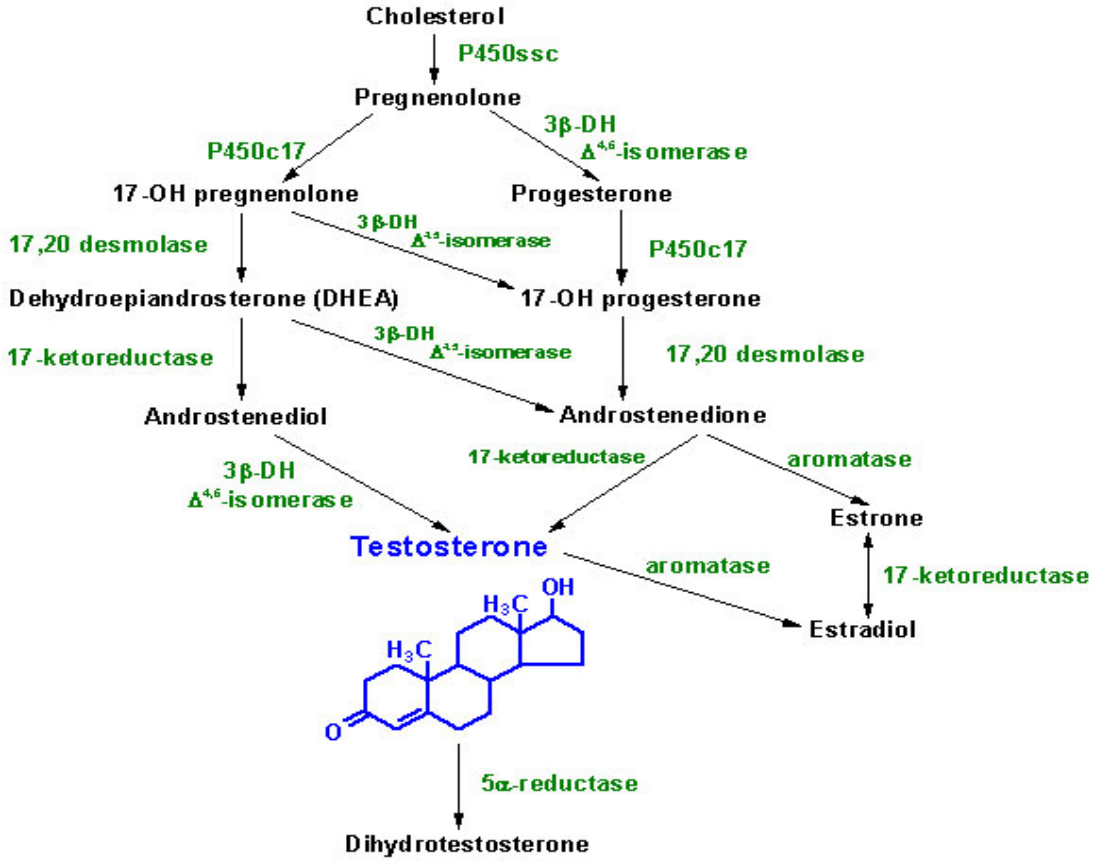

Figure 1.3: Steroidogenesis pathway in vertebrates (source: http://www.indstate.edu/thcme/mwking/steroid-hormones.html)

decreased production of androgens as well as estrogens, as androgen levels may negatively control their own production (Figure 1.1). Metabolites of androstenedione produced through fermentation processes involving *Myrothecium stiatisporum* have shown aromatase inhibition as well (Mahato and Garai, 1997). *In vitro* aromatase inhibition has also been seen in male and female rats that were fed PCB-contaminated lake trout (Gerstenberger, 2000). PME has been shown to decrease 17-β-estradiol levels in female white sucker ovarian follicles without affecting testosterone levels, and thus has been implicated in aromatase inhibition (McMaster *et al.*, 1996). PME has also been found to increase aromatase activity

in vitro using mosquitofish brain tissue (Orlando *et al.*, 2002). However, this may not be unexpected as aromatase inhibition has been shown to cause increases in aromatase mRNA and enzyme (Harada *et al.*, 1999), and therefore tissue *in vitro* from organisms exposed to aromatase inhibitors may show increased aromatase activity. Direct evidence of the aromatase inhibition potential of PME is lacking.

1.4 *In vitro* sex steroid analysis

Endocrine disruption may occur at several different levels along the hypothalamous-pituitary-gonadal axis. The most obvious results are displayed with decreased steroid hormone levels. Low levels of steroid hormones in the organism are due to low production of steroid hormones by their respective gonads. McMaster et al (1995) have devised a protocol for measuring steroid hormone production in fish gonadal tissue *in vitro*. These methods have been used successfully for the *in vitro* measurement of gonadal steroid production for mummichog (Hewitt *et al.*, 2002), white sucker (McMaster *et al.*, 2001) and goldfish (McCarthy *et al.*, 1997). Information on alterations to gonadal steroid production along with androgen receptor binding and aromatase inhibition data give a better overall assessment of the impact(s) a particular effluent may pose to the organism.

Gonadal tissue from exposed fish must first be removed from the organism and incubated in specialized buffer (Medium 199 or M199) (McMaster *et al.*, 1995). A proportion of steroids produced in the tissue during incubation will leach out into the buffer. A subsequent aliquot of the media after incubation can then be analyzed for its testosterone and 17-β-estradiol by radioimmunoassay

(RIA). The ability to perform an RIA depends on the availability of: i) an antiserum specific to the steroid to be measured, ii) a radiolabelled form of the steroid, iii) pure, unlabelled steroid for the assay standards, and iv) a method by which antibody-bound steroid can be separated from free steroid (McMaster *et al.*, 1995). For the assay procedure, a fixed concentration of radiolabelled steroid is incubated with a constant dilution of specific antiserum such that the antibody will bind only 50% of the total steroid concentration. Upon addition of unlabelled steroid, there will be competition between labeled and unlabelled steroid for a limited number of antibody sites (Figure 1.4). Thus, the amount of radiolabelled steroid bound to the antibody will decrease as the concentration of unlabelled steroid increases. The concentration of bound radiolabelled steroid is inversely proportional to the amount of unlabelled steroid present. The concentration of steroid present in the aliquot of media buffer from the incubation of gonadal tissue can be determined by comparison with the results obtained using known concentrations of unlabelled steroids to generate a standard curve.

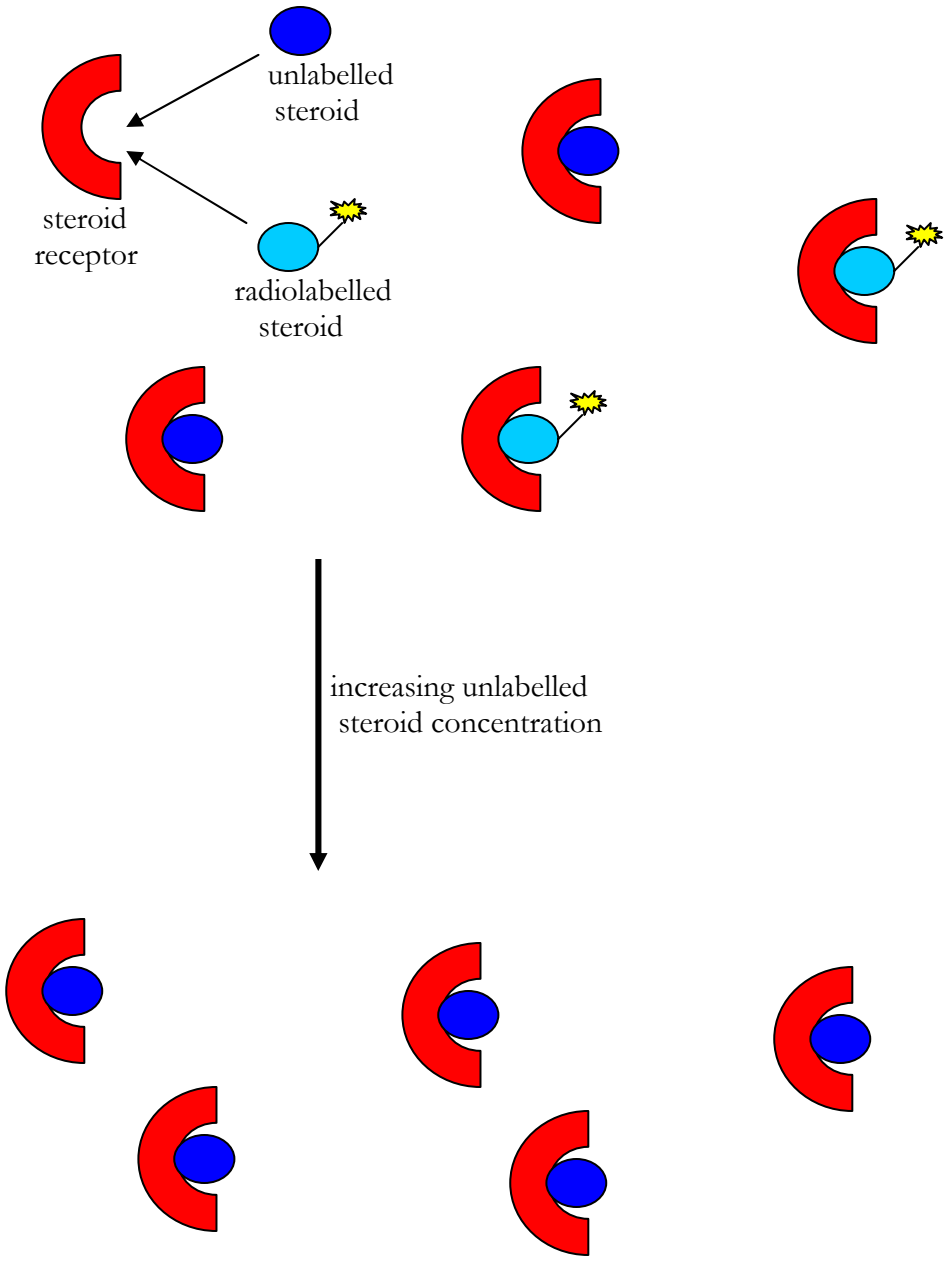

Figure 1.4: Competitive displacement of radiolabelled hormone by increasing unlabelled hormone concentration. Unbound radiolabelled hormone is removed by charcoal-stripping before measuring radioactivity of the assay mixture

1.5 Mosquitofish

Gambusia affinis, also known as the western mosquitofish, is a member of the Poeciliidae family which is of the order Cyprinodontiformes. The generic name *Gambusia* is reflective of the mosquitofish's small size as it is derived from "Gambusina", a term commonly used in Cuba meaning "small" or "of no importance" (Kuntz, 1913). They are a viviparous (live-breeding) freshwater species that feed on small insects and their larvae as well as small crustaceans and plant material. *G. affinis* are indigenous to the southern United States (Krumholtz, 1946), but have been introduced to many areas including Europe and the North Island of New Zealand as a mosquito-control measure.

Mosquitofish are sexually dimorphic. Females tend to be larger and have a dark spot toward the posterior end on their bellies when gravid. The most telling characteristic between male and female mosquitofish is the differential development of the anal fin. Females have an undifferentiated anal fin, roughly triangular in shape with rounded edges and consisting of ten bony rays. Sexually mature males have a differentiated anal fin. Rays 3, 4 and 5 are elongated into a structure known as a gonopodium, which serves as an intromittent device to deliver sperm to female mosquitofish genitalia. The tip of the gonopodium is further differentiated with hooks and serrae to facilitate copulation. Figures 1.5 and 1.6 below display images of sexually mature female and male *G. affinis* respectively.

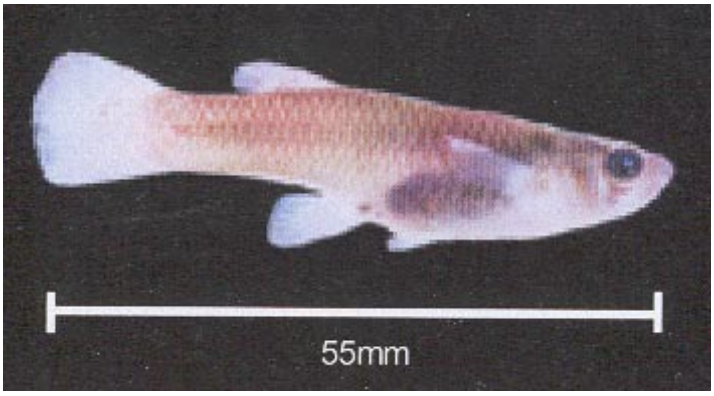

Figure 1.5: Sexually mature female *G. affinis*

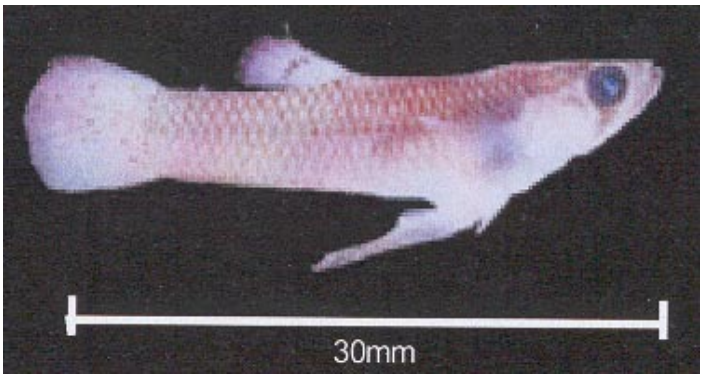

Figure 1.6: Sexually mature male *G. affinis*

Figure 1.6 shows the gonopodium in a resting position. The gonopodium swings forward laterally to a copulatory position through the action of enlarged and specialized muscles (Rosen and Tucker, 1961). Gonopodium action is further supported by a suspensorium consisting of a series of complex internal bony supports that are anchored to the axial skeleton (Rosen and Tucker, 1961).

Gonopodial development has been induced in female mosquitofish through the addition of androgenic compounds such as methyltestosterone and ethynyltestosterone (Turner 1941a, 1941b, 1941c), androstenedione and

androstanol (Husinger and Howell, 1991), and 11-ketotestosterone (Angus *et al.*, 2001). Turner (1941b) found that the ability of methyltestosterone and ethynyltestosterone to induce gonopodial development in female mosquitofish decreases with increasing age of the fish and also that these compounds induce precocious gonopodial development in males. The induced gonopodium in females does not regress when androgen administration stops (Turner, 1941a), although normal female anal fin development will reoccur if the induced gonopodium is excised (Turner, 1960). There is no evidence of hermaphrodism in treated females (Turner, 1941a) and it is thus believed that gonopodium development in mosquitofish is under androgenic control. However, estrogens are known to play a role in bone tissue generation, thus their role and the role of aromatase (if any) in the development of mosquitofish gonopodia remains to be elucidated.

Mosquitofish have been shown previously to be an indicator of the androgenic potential of effluents in receiving water systems as shown by an elongation of the female anal fin, signifying gonopodium development (Bortone and Cody, 1999; Cody and Bortone, 1997). They have been used in laboratory studies to determine androgenic potential of PME (Ellis *et al.*, 2003; Drysdale and Bortone, 1989) and microbial degradation products from plant sterols (Howell and Denton, 1989; Denton *et al.*, 1985). Mosquitofish have also been exposed to exogenous androgens in laboratory assays to study their effects on anal fin elongation in the female species (Husinger and Howell, 1991). In all these cases, the elongation of the female anal fin was made by visual inspection or caliper measurements and compared with the standard lengths of the fish. Recently, a

quantitative study using computer image analysis has been developed to measure gonopodial development in male and androgen-treated female mosquitofish (Angus *et al*., 2001). The anal fin of *Gambusia affinis* contains 10 skeletal rays that impart its structure (Turner, 1941b). Gonopodium development in males involves the elongation of rays 3, 4 and 5. Females have also been shown to display elongation of rays 3, 4 and 5 upon exposure to androgens (Turner, 1941b; Husinger and Howell, 1991). Angus *et al* (2001) have shown that using computer image techniques, the 4:6 ray length ratio of the anal fin can be a determinant of mosquitofish exposure to androgens. Figures 1.7 and 1.8 on the following page show anal fin images from a control female mosquitofish and a female mosquitofish exposed to methyl-testosterone. The 4:6 anal fin ray ratio for normal sexually mature females is 1:1 and for normal sexually mature males it is about 2.5:1 (Angus *et al*., 2001). Anal fin rays can be measured from digital images of mosquitofish anal fins using Image Tool software available for free from the University of Texas Health Sciences Center at San Antonio (http://ddsdx.uthscsa.edu/dig/download.html). Log-transforming the 4:6 ratio data provides normal distribution and allows the use of parametric statistical analysis (Angus *et al*., 2001). Elongation of rays 3, 4 and 5 in female mosquitofish are the first and most obvious response to exposure to androgens (Turner, 1941b). Thus it is possible to postulate that significant differences in 4:6 anal fin ray length ratios suffice to indicate mosquitofish exposure to environmental androgens.

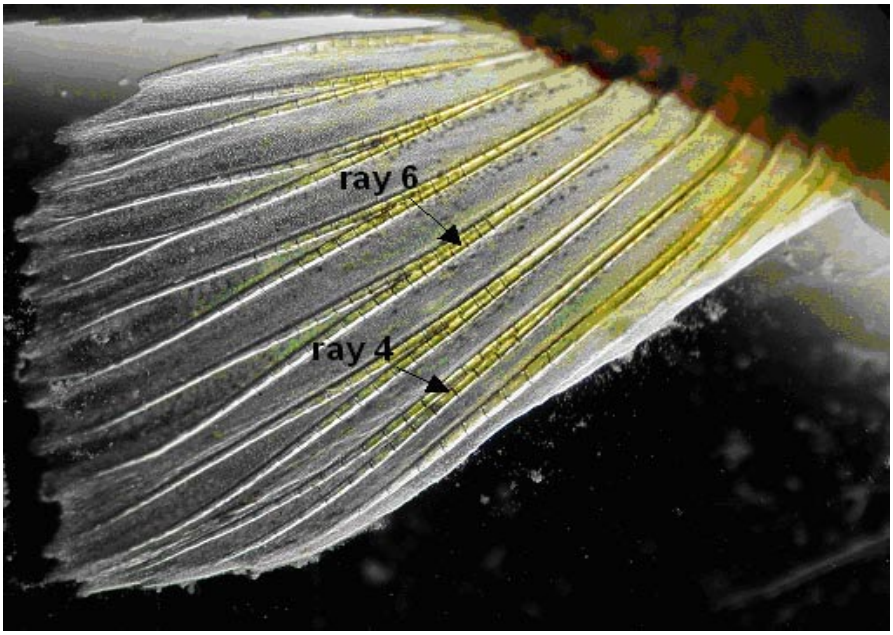

Figure 1.7: Anal fin of control female mosquitofish

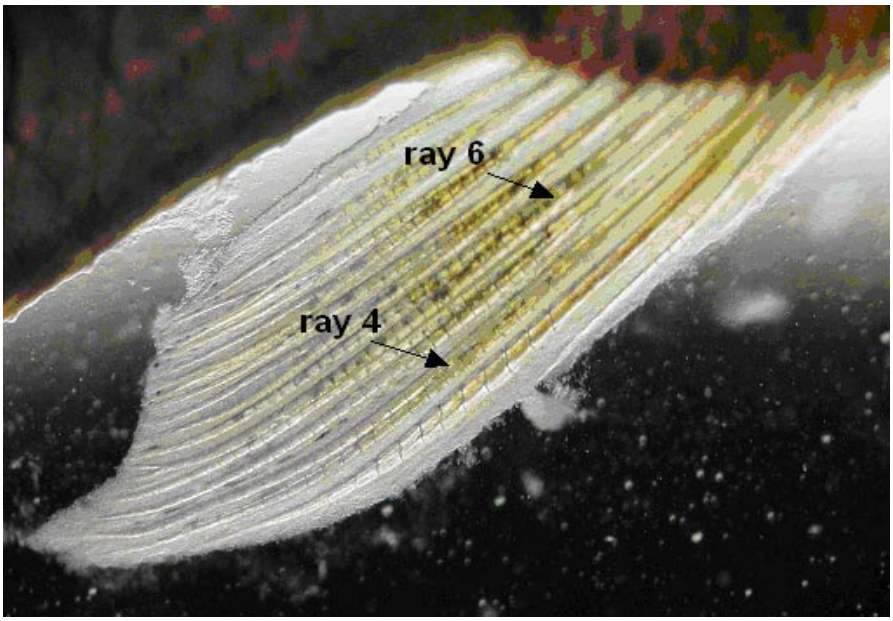

Figure 1.8: Anal fin of mosquitofish exposed to methyl-testosterone

1.6 Endocrine disrupting potential of effluents

1.6.1 Pulp-mill effluent (PME)

There has been extensive study on the effects of PME entering receiving water environments over the past two decades. Many of these studies focus on measures of reproductive impairment in fishes such as decreased egg and gonad size, reduced fertilization and hatching success (McMaster *et al.*, 1996; Munkittrick et al., 1992a, 1994); disrupted fecundity-weight relationships (Gagnon *et al.*, 1994a); decreases in serum sex steroid levels and overall gonadal steroid production (Van Der Kraak *et al.*, 1992; Munkittrick *et al.*, 1992b; McCarthy *et al.*, 1997); and changes in population dynamics (Kovacs *et al.*, 1997; Munkittrick *et al.*, 1997). Through substantial process and treatment improvements, the pulp and paper mill industry has alleviated many toxicity issues with PME in relation to fish health (Kovacs *et al.*, 1997; Swanson *et al.*, 1994; Kloepper-Sams *et al.*, 1994a, 1994b). However, those changes were generally targeted at reducing toxicity, BOD loading and organochlorines, and reproductive impacts have still been observed at some sites (Munkittrick *et al.*, 1992b, 1992c; MacLatchy *et al.*, 1997) with decreased gonad size continuing to be a response seen in fish exposed to PME at some sites in Canada (Munkittrick *et al.*, 2002). Differences in aquatic habitats and type of receiving water (river, estuary, marine or lake) make generalizations about these effects difficult and it is suggested that responses be qualified on a site-by-site basis (Munkittrick *et al.*, 2002).

Estrogenic activity of PME has been reported in studies involving vitellogenin gene expression induction in caged whitefish (Mellanen *et al.*, 1999), and induced vitellogenin plasma levels in immature rainbow trout (Tremblay and

Van Der Kraak, 1999). The plant phytosterol β-sitosterol has been implicated in producing these effects (Tremblay and Van Der Kraak, 1998) as it is a constituent of PME. Other PME compounds such as lignans, stilbenes, resin acids, and other sterols have shown weak estrogenic activity as well (Mellanen *et al.*, 1996). However, a true consensus on the compound(s) responsible for these effects is lacking. Furthermore, some studies have failed to show any estrogenic effects with PME (van den Heuvel *et al.*, 2002; Van Der Kraak *et al.*, 1998b).

Androgenic activity of PME has mostly focused on alterations in secondary sexual characteristics of mosquitofish, namely the induction of gonopodial development in females (Bortone and Cody, 1999; Cody and Bortone, 1997; Drysdale and Bortone, 1989; Howell *et al.*, 1980). Other androgenic responses believed to be caused by PME include American eels with precocious testicular development and enlarged eyes (Caruso *et al.*, 1988) as well as a significant male sex ratio bias in eelpout embryos (Larsson *et al.*, 2000). Other masculine characteristics in female mosquitofish exposed to PME include dorsal and pelvic fin height, eye diameter and body depth when compared to reference fish (Bortone and Davis, 1994). Gonopodial development in female mosquitofish has been seen in PME from a combined thermomechanical/kraft process (Ellis *et al.*, 2003) as well as a kraft process alone (Cody and Bortone, 1997). Androstenedione (AD) has been detected in a Florida river receiving pulp and paper mill discharge, theoretically due to the microbial degradation of plant sterols, and has been implicated in masculinizing the female mosquitofish population (Jenkins *et al.*, 2001). However, a subsequent study using assays with cells transfected with human androgen receptor and reporter gene constructs

showed that the active fractions from the same river samples did not contain androstenedione (Durhan *et al.*, 2002). An earlier study has shown masculinization of female mosquitofish in controlled exposures to β-sitosterol in the presence of the bacteria *Mycobacterium smegmatis* (Howell and Denton, 1989). Although *M. smegmatis* can biotransform β-sitosterol to both AD and 1,4-androsta-diene-3,17-dione (ADD) with respective yields of 13% and 30% (Mahato and Garai, 1997), there has been relatively little attention paid to the potential effects of ADD. Also, there are no known studies on the potential anti-androgenic mechanism of PME, even though there are studies showing decreased steroid serum levels in exposed fish (Van Der Kraak *et al.*, 1992; Munkittrick *et al.*, 1992b).

Aromatase inhibition has also been postulated as a mechanism for masculinization of female mosquitofish exposed to PME. With its inhibition, it is believed that an excess of testosterone in the female may cause an androgenic response (i.e. anal fin elongation). To date relatively few studies have examined this theory. Recently, a study measured the aromatase activity in ovarian and brain tissue from female mosquitofish collected downstream from a Florida pulp mill (which had previously showed masculinization effects) and showed increased aromatase activity in both tissue samples (Orlando *et al.*, 2002). The authors attribute these results to mosquitofish resilience in contaminated conditions and conclude that aromatase inhibition is not a mechanism of masculinization. Although anal fin rays between the exposed female mosquitofish and female mosquitofish collected from a reference site showed no significant difference, the exposed fish had a significantly higher number of ray segments in their anal fins.

This may also be an indication of an androgenic response in mosquitofish (Turner, 1941b). Also, as mentioned previously, an aromatase inhibition response *in vitro* using tissue from exposed fish may include increased aromatase activity due to the ability of aromatase inhibitors to cause an increase in aromatase mRNA and enzyme (Harada *et al.*, 1999). More work is needed to elucidate the potential of PME to cause aromatase inhibition, androgenic and anti-androgenic responses in fish.

1.6.2 Sewage treatment-plant effluent (STP)

Sewage treatment-plant effluent (STP) was first implicated in endocrine disruption in fish with the detection of the female egg yolk protein vitellogenin (VTG) in male rainbow trout caged downstream from STP discharges in England (Purdom *et al.*, 1994). Subsequent studies have shown that other rivers in the United Kingdom receiving STP discharge are inducing VTG in male or immature fish (Harries *et al.*, 1996; Tyler and Routledge, 1998). Similarly, studies from France (Flammarion *et al.*, 2000) and the United States (Folmar *et al.*, 1996) have found VTG induction in male chub and male carp downstream of municipal sewage discharges. VTG induction is caused by natural and synthetic pharmaceutical estrogens as well as other environmental estrogens such as nonylphenol and its associated ethoxylates occurring primarily from industrial processes such as textile manufacturing (Sheahan *et al.*, 2002). Other endocrine-disrupting responses from STP include inhibition of testicular growth in male rainbow trout (Harries *et al.*, 1997) and the prevalence of ovarian tissue in male gonads (ovotestis) (Jobling *et al.*, 1998). 17-β-estradiol, estrone and 17α-ethyinyl

estradiol were all detected in STP samples from seven treatment plants in the United Kingdom, ranging from 80 ng/L for the natural hormones and 7.0 ng/L for the synthetic ones (Desbrow *et al*., 1998).

The effect of STP on gonpodial development in mosquitofish has been examined. In Australia, significantly reduced lengths of gonopodia in male eastern mosquitofish (*Gambusia holbrooki*) were found in fish collected from a tributary downstream of a municipal sewage treatment plant compared with those collected upstream (Batty and Lim, 1999). They also found no significant difference in the presence or absence of spermatozeugmata (sperm packets) between the sample groups, which suggests that spermatogenesis may not have been affected. However, whether all the male mosquitofish in the study were sexually mature is unknown. Full development of the gonopodium in male mosquitofish requires 30 to 60 days and its complete development is noted with the presence of hooks and spines on the gonopodial tip (Angus *et al*., 2001). A study from the USA showed no significant difference in gonopodial development between groups of male western mosquitofish (*Gambusia affinis*) sampled from a river downstream from a municipal wastewater treatment plant and another discharge-free river site (Angus *et al*., 2002). The study also failed to show the presence of VTG in mosquitofish blood samples from all sites. In both mosquitofish studies mentioned, gonopodium development was measured as an extension of the 3-4-5 anal fin rays indexed against total length of the fish. One further study looked at the effect of 17-β-estradiol on the gonopodial development and sexual activity of *G. holbrooki* (Doyle and Lim, 2002). Using a flow-through laboratory setup, juvenile male mosquitofish showed a significant

decrease in gonopodium length (including anal fin ray 4 to 6 ratio), percentage of hooks found on the distal tip of the gonopodium and copulatory attempts with females in a dose-dependent manner (concentration used 20, 100 and 500 ng/L). Significant decreases in these endpoints suggest possible effects on the reproductive success for the species when exposed to high enough concentrations of estrogens.

1.6.3 Isolation and identification of endocrine-disrupting compounds in effluents

There are a large number of chemical and organic compounds in wastewater such as PME, and it is very difficult to identify and evaluate them all individually. PME contains a range of compounds including dissolved and colloidal organics, lignin compounds, chlorinated compounds from bleaching processes and dissolved inorganics (i.e. NaOH) (Sreekrishnan, 2001). PME also contains phytosterols that come from wood and have been shown to induce androgenic responses in female mosquitofish when microbially degraded (Howell and Denton, 1989). STP has not been well characterized, but has been shown to contain estrogens and estrogenic compounds (Desbrow *et al.*, 1998). Sterols and steroids are relatively low in molecular weight (200-400 g/mol) and hydrophobic (IPCS, 2002). They tend to stay adsorbed onto suspended organic material in wastewaters (IPCS, 2002).

Isolating bioactive fractions of wastewaters is a major challenge. Many recent studies have taken a toxicity identification and evaluation (TIE) approach in trying to isolate a biologically active fraction or component of complex effluents (Hewitt *et al.*, 2000; Parrot *et al.*, 2000; Desbrow *et al.*, 1998; Jenkins *et al.*,

2001). In these studies, solid-phase extraction and reverse-phase HPLC methods were used to help isolate bioactive fractions from PME and STP. There are some problems inherent with these methods that may affect the potencies of the effluents and/or efficiency of isolation: i) sample handling, storage and preparation, ii) high molecular weight interferences with investigations of low molecular weight compounds, and iii) the complexities of low molecular weight compounds in wastewaters. Therefore, when trying to isolate bioactive compounds in effluents, an extraction method should be designed to: i) allow for quick processing of the effluent sample so as to minimize or eliminate storage time, ii) target low-molecular weight extractives, particularly the moderately non-polar fraction where potential steroidal compounds would reside, and iii) be low-cost and easy to use in field work.

XAD resins have been used in the extraction of organics from water samples (Ohno, 2002; Malcom et al., 1992) and pulp mill effluent (Santos *et al.*, 2000) quite effectively. In particular, XAD-7 resin is specific for steroids and steroid-like compounds. It has been used to extract testosterone from fermentation batch reactors (Mahato and Garai, 1997). XAD resins are non-ionic exchange resins and are preferable over anionic resins for extraction of organics due to the former's better-reported recoveries (Malcom *et al.*, 1992). Glass-fiber (GF) filters allow for entrapment of suspended organic matter in the effluent, with GF-C and GF-F filters having 1.5 and 0.45 μm pore sizes respectively. The use of XAD-7 resin in combination with GF-C and GF-F filters could theoretically trap a proportion of the sterols and steroids potentially present in an effluent sample.

1.7 Research objectives

The purpose of this thesis is to examine the androgenic potential of several effluents using specific, sensitive and quantitative methods. The effluents include: a New Zealand TMP/kraft pulp and paper mill, a New Zealand STP and a Canadian TMP/kraft pulp and paper mill. The methods include an *in vivo* assessment using a whole-organism bioassay (mosquitofish) with quantifiable analysis (anal fin ray 4 to 6 ratio), and a suite of *in vitro* analyses that include a fish androgen receptor assay, fish aromatase inhibition assay and gonadal tissue steroid analysis by radioimmunoassay. For the Canadian PME, only the *in vitro* fish androgen receptor assay and fish aromatase inhibition assay were done, as an *in vivo* analysis with mosquitofish has already been performed (Hardy, 2002). Currently, there is no accepted standard method or procedure for the detection of endocrine-modifying compounds. From the many different individual assays that exist for detecting and screening endocrine disruptors, there is no consensus on the interpretation of individual test results with respect to ecological health. There is also no legal provision at this time preventing the discharge of endocrine disrupting compounds into receiving environments. The approach taken in this thesis will provide a more comprehensive (*in vivo* and *in vitro*) assessment of the androgenic potential of the effluents. An analysis of the effluents' ability to bind to the fish androgen receptor, inhibit fish aromatase activity, decrease steroid production in gonadal tissue, and induce anal fin ray elongation in female mosquitofish compared to respective upstream samples as reference, will provide data to make this assessment.

Other experiments in this thesis will also look at the androgenic potential of ADD, a compound that could potentially be found in PME, using the fish androgen receptor assay and female mosquitofish bioassay. The potential *in vivo* anti-androgenic effect of the mammalian anti-androgen cyproterone acetate will be examined by studying its ability to inhibit anal fin elongation in the mosquitofish bioassay. Also, the potential anal fin elongation due to aromatase inhibition in female mosquitofish using HAD will be examined. The hypotheses tested in this study are:

1) There is no difference between the androgenic response potential of the effluent (NZ PME, Canadian PME, and NZ STP) samples and their respective upstream reference/blank samples.
2) There is no difference between the androgenic response potential of PME and STP.
3) Every fish-based *in vitro* assay indicates the same response for the same androgen.
4) There is no difference between the *in vivo* and *in vitro* androgenic response potential of the effluents.

2.0 EXPERIMENTAL METHODS

2.1 Description of mills and collection sites

The New Zealand pulp mill from which effluent was sourced for this study was situated in Kawerau on the North Island. It is an integrated bleached kraft and thermomechanical (TMP) pulp and paper mill. It primarily used softwood (*Pinus radiata*) furnish. The bleaching process used sodium hypochlorite and/or chlorine dioxide. A moving bed biofilm reactor pre-treated the TMP wastewater before it is combined with the bleached kraft mill effluent. Secondary treatment consisted of a three-pond aerated stabilization basin with effluent retention time of 5 to 6 days. The effluent was discharged from the secondary treatment system into the Tarawera River at a mean volume of 180,000 m^3/day and an average temperature of 26 to 28^oC. The effluent dilution in the Tarawera River ranged between 5 and 12%. Tarawera River samples used for upstream reference were collected well upstream of the Tasman outfall and at least 500 m upstream from other discharge points of effluent.

The Canadian pulp mill from which effluent for this study was obtained was located in Thunder Bay, Ontario and also was a combined bleached kraft/TMP mill. Mill furnish consisted of jackpine (60-70%) and spruce (30-40%). Chlorine dioxide was used in the bleaching process. Effluents from the TMP and kraft processes were settled in primary clarifiers before they were combined and sent to an activated-sludge stirred-tank reactor. Retention time in the activated sludge reactor was approximately 3 hours and typically had a mixed-liquor suspended solids value of 4500 mg/L. The treated effluent was discharged into the Kaministiqua River. Effluent dilution in the Kaministiqua River ranged

between 6 and 13%. Kaministiqua River samples used for upstream reference were collected approximately 2 km upstream from the discharge point.

The New Zealand STP was located in Rotorua on the North Island. It handled approximately 18,000 m^3 of raw municipal sewage from a population of about 58,000. Pre-treatment of the sewage consisted of screening and grit removal. Primary treatment consisted of sedimentation tanks. An activated sludge process for secondary treatment then treated the effluent before it moved on to the final clarifiers. The effluent was then pumped from the clarifiers to two holding ponds where eventually it was sprayed over the nearby Whakarewarewa forest. Therefore, no appropriate "upstream" reference was available.

2.2 Extraction apparatus and procedure

In the experimental extraction procedures, XAD resins were used in a low-pressure chromatography column. Please refer to Figure 2.1 for a flowchart representation of the following described procedure. In these experiments, 5 L effluent and upstream river samples were GF-C and GF-F filtered, treated with HCl to pH 4 (to ensure protonation of all hydroxylated compounds) and then pumped through a column (2.5 cm diameter, 40 cm in length) packed with 25 g of clean XAD-7 resin at a rate of 10 mL/min, such that the sample was processed within the same day of collection. A blank sample was also performed with 5 L of deionized water according to the procedure just described. The entire apparatus was easily transportable and was amenable to on-site processing, as it required little space. The effluent sample volume (5 L) was chosen so as to avoid

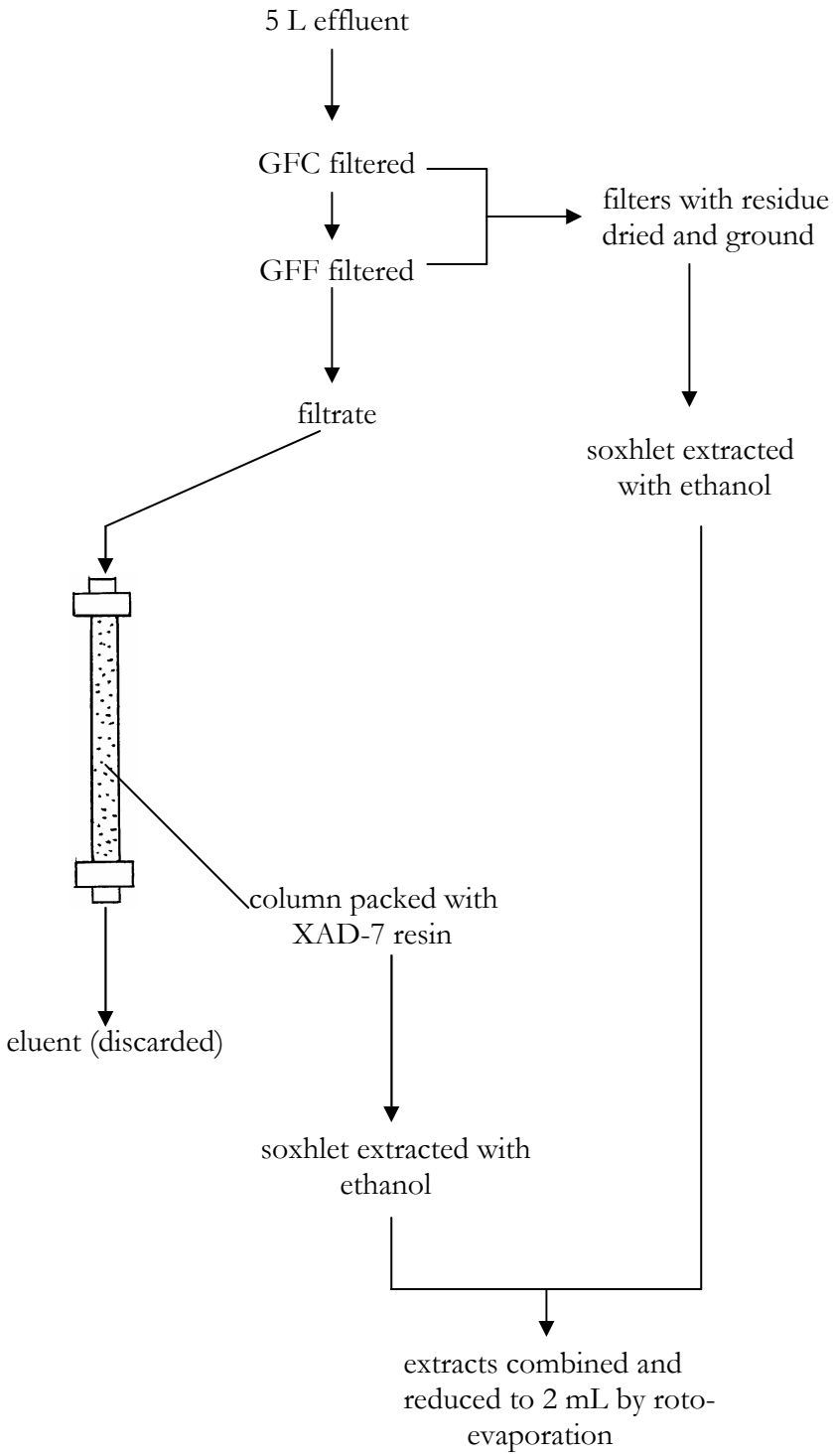

Figure 2.1: Schematic flowchart of extraction procedure

saturation of the XAD resin. The use of a larger column in order to increase resin capacity (and therefore increase effluent sample volume) was avoided due to the fact that larger columns promote band-broadening, which may cause loss of analyte (Skoog and Leary, 1992). Band-broadening may be reduced by decreasing the flowrate of the effluent being pumped through, but this would have increased the time required to process the sample considerably and introduce problems of effluent stability that were designed to be avoided.

The XAD-7 was cleaned according to the methodology outlined in Ohno (2002): five batch extractions with 0.1 N NaOH stirred for 1 hour were followed by three repeated soxhlet extractions with methanol for 4 hours. Once packed into the column, the resin was eluted with 150 mL each of 0.1 N NaOH, 0.1 N HCl and deionized H_2O. The UV-absorbance of the deionized H_2O eluent between 200 nm and 800 nm was found to be zero, indicating effective purification of the resin. Once the sample was passed through the column, the resin was removed and allowed to dry in a desiccator. The dry sample resin and the GF-C/GF-F filters were then soxhlet extracted separately with 150 mL of ethanol overnight. The ethanol extracts were combined and reduced in volume by rotary evaporation to 2 mL and stored in a refrigerator until further use. GC-MS analysis of all extracts was performed using a HP 5890 series II gas chromatograph with a HP 7673A auto sampler connected to a HP 5971A mass selective detector (MSD) via a 50 m HP Ultra-2 column with helium as a carrier gas. The MSD was operated in scan or selected ion-monitoring mode (SIM). All quantitations were undertaken using HP EnviroQuant software and a full suite of standard steroid compounds (testosterone, 17-β-estradiol, androstenedione, 1,4-

androsta-diene-3,17-dione) for calibration. Samples were derivatised by silylation (BSTFA) prior to GC-MS analysis.

2.2.1 Validation of extraction method

The ability of the resin to retain organics was determined by measuring the colour and total carbon portion of the effluent after the passage of each one of the five litres of effluent sample. A 20 mL eluent sample from the resin column was taken and analyzed for colour following the NCASI method for measuring mill effluent and receiving water colour. Another 20 mL eluent sample was taken and analyzed for total organic carbon using a Shimadzu Total Organic Carbon Analyzer Model TOC-5000. Table 2.1 shows the percentage of retention as compared to an effluent sample that has not been passed through the resin column.

Table 2.1: Retention of colour and total carbon by XAD-7 resin from effluent sample

Volume passed through column (L)	Percentage of total carbon retained (%)	Percentage of colour retained (%)
1	58.3	68.5
2	53.6	64.8
3	54.0	63.0
4	55.3	63.0
5	52.2	55.6

A 5-L treated effluent sample was also spiked with a 50 μg methyl-testosterone surrogate and passed through a fresh XAD-7 resin column. The resin was then soxhlet extracted with ethanol. The ethanol extract was reduced in volume to 2 mL and analyzed for methyl-testosterone content by GC-MS. The

resin was found to retain 41.5% of the methyl-testosterone surrogate. This compares agreeably (39.5-41% recovery of 17-β-estradiol cypionate) with a recent method designed to remove steroids from PME using solid-phase extraction and HPLC (Jenkins *et al.*, 2001).

2.3 Androgen receptor assay

The androgen receptor assay used herein is a hybrid of those presented in Wells and Van Der Kraak (2000) and Sperry and Thomas (1999). The androgen receptor protein was isolated from sexually mature rainbow trout brain tissue. Rainbow trout is commonly found in New Zealand and Canadian waters and its physiology is well studied compared to other species. Steroid hormone binding may be represented by the reversible reaction:

$$R + H \leftrightarrow RH \qquad (1)$$

where R is receptor and H is hormone. At equilibrium, it can be described as:

$$K_D = \frac{[R][H]}{[RH]} \qquad (2)$$

where K_D is the dissociation constant (Lodish *et al.*, 1995). The K_D value is equivalent to the concentration of hormone at which one-half of the receptors contain bound hormone (Lodish *et al.*, 1995). The K_D value can also be interpreted as a measure of the affinity of the receptor for the hormone. The

lower the K_D, the higher the affinity of a receptor for the steroid. K_D values for steroid receptors are usually determined in order to show appropriate selectivity of the receptor protein preparation (i.e. how well and whether the protein binds its appropriate hormone). K_D values were established by competitive radioligand binding studies (please see Figure 1.4 for a diagram on competitive radioligand displacement).

The rainbow trout androgen receptor protein was then exposed to varying concentrations of radiolabelled testosterone (^{3}H-testosterone) along with either an excess of unlabelled testosterone (to determine the non-specific binding to proteins other than receptors) or excess buffer with no testosterone (to determine the total binding). Subtraction of the non-specific binding values from the total binding values at their respective ligand concentrations provides specific binding values (Figure 2.2). The resulting specific binding saturation data of the androgen receptor was analyzed by non-linear regression using GraphPad Prism 3 software in order to determine the K_D value. The K_D value for the nuclear-fraction rainbow trout brain androgen receptor was found to be 2.27 nM which comparably agrees with those values found by Sperry and Thomas (1999) for nuclear-fraction atlantic croaker androgen receptor (1.1 nM) and Wells and Van Der Kraak (2000) for cytosolic fraction rainbow trout brain androgen receptor (1.43 nM). The specificity of the assay is validated by the fact that the non-specific binding is below 50% of the total binding at all concentrations (Sperry and Thomas, 2000).

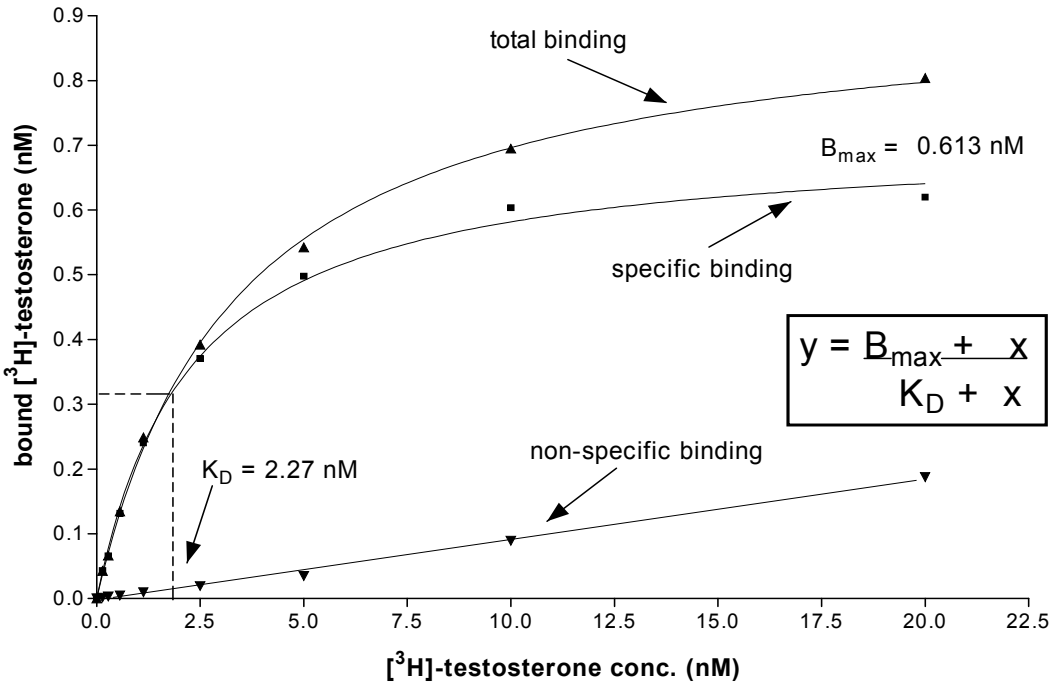

Figure 2.2: Saturation binding data of ^{3}H-testosterone to rainbow trout brain androgen receptor

2.3.1 Tissue preparation

Tissue preparation was performed according to Sperry and Thomas (1999) with the exception of the addition of a commercial protease inhibitor (Pefabloc SC) in order to increase stability of the receptor protein during storage. Excised rainbow trout brains were preserved in buffer with 50 mM Tris-HCl, 1 mM NaEDTA, 30% glycerol and 0.5 mM of commercial protease inhibitor (Pefabloc SC). The tissue samples were stored until use at –80°C. When ready for the next stage, the tissue samples were thawed and homogenized with four passes of a tissue grinder. The samples were pooled and charcoal-stripped to remove endogenous steroids. Using the same buffer and including 1% activated charcoal and 0.1% dextran (by weight), the samples were centrifuged at 10,000 g

for 15 minutes. The supernatant was collected and immediately ultracentrifuged at 100,000 g for one hour. The resulting supernatant containing the cytosolic fraction of androgen receptor was collected and stored at –80°C. The crude nuclear pellet was washed one time with the same homogenizing buffer (without charcoal and dextran) with the addition of 0.7 M KCl, re-suspended and then incubated in the buffer for 20 minutes. The re-suspended nuclear pellet was ultracentrifuged again at 100,000 g for one hour. The resulting supernatant was discarded, the nuclear pellet re-suspended in fresh buffer and stored at –80°C until further use.

2.3.2 Assay procedure

The assay procedure follows the methods outlined in Wells and Van Der Kraak (2000). The assay involved creating a reaction mixture of receptor, radiolabelled hormone and endogenous steroid or steroid analog. Please refer to Figure 2.3 on the following page for a flowchart representation of the assay procedure. The reaction was carried out in 5 mL borosilicate glass tubes containing 50 μL of ^{3}H-testosterone, 50 μL of steroid and 150 μL of diluted (1:2 in 50 mM Tris-HCl, 1 mM NaEDTA, 10% glycerol (TEG) buffer) nuclear androgen receptor preparation. The final concentration of ^{3}H-testosterone in the reaction mixture was 1.25 nM and the final concentration of steroid ranged from 0.01-1000 nM (logarithmic scale). Where effluent extracts were used, 50 μL of TEG buffer was added along with 5 μL of effluent extract (in ethanol). The final concentration of ethanol in the reaction tube was 2% by volume, which has not been found to interfere with ligand-receptor binding (Wells and Van Der Kraak,

2000). Unless incubated at specific temperature or in centrifugation tubes, samples were at all times kept on ice.

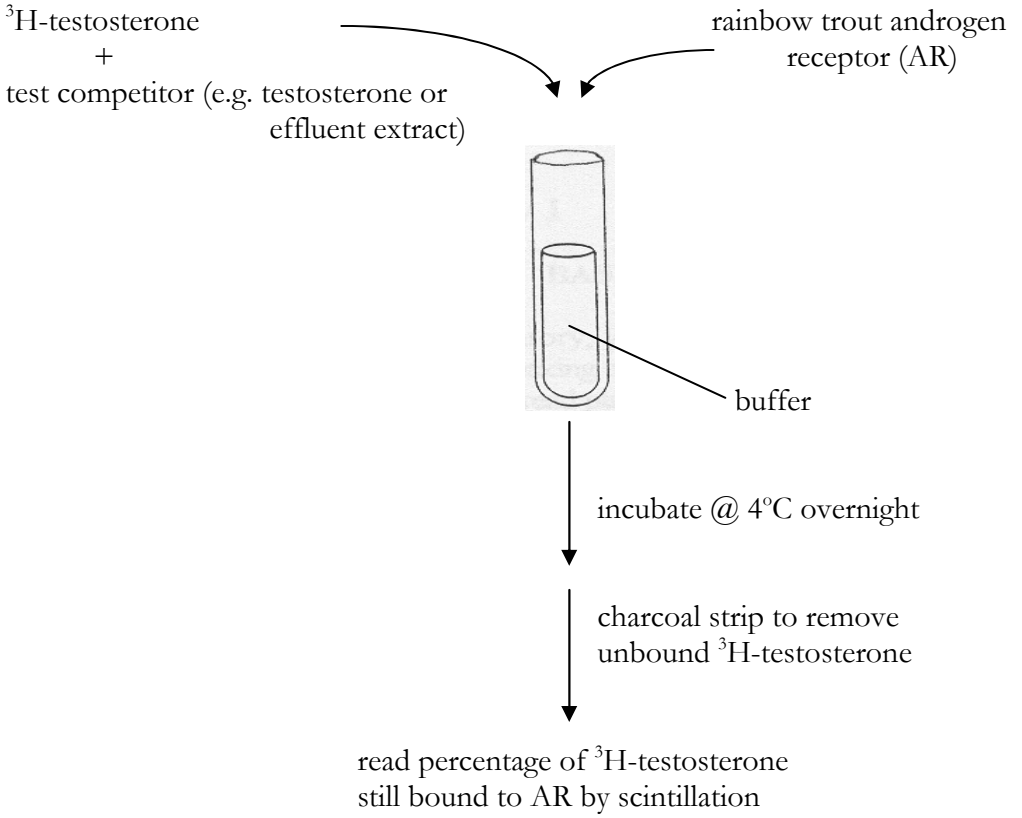

Figure 2.3: Flowchart representation of the rainbow trout androgen receptor binding assay

Steroids were added first to the tubes followed by nuclear androgen receptor. The tubes were vortexed and incubated overnight at 4°C. After incubation, 500 μL of a 0.5% activated charcoal/0.05% dextran – TEG buffer solution was added to each tube. The tubes were allowed to incubate for 5 minutes and then centrifuged at 2000 g for 15 minutes to remove unbound

ligands. The supernatant from each tube was collected into separate 5 mL scintillation vials to which was added 5 mL of scintillation cocktail (2 L toluene, 1 L Triton X-100, 12 g 2,5-diphenyloxazole, 0.6 g 1,4-Bis[2-5-Phenyloxazoly]Benzene) (McMaster et al., 1992). The vials were then measured with a scintillation counter and the counts for each vial were recorded. A blank tube set provided a blank value (no receptor or ligand) that was subtracted from all values. Another tube set containing only receptor and radiolabelled ligand (and 50 μL of TEG buffer) provided a count that represented 100% binding for the radiolabelled ligand. All counts were divided by this value in order to normalize binding data in terms of percentage of bound radiolabelled ligand. Effluent extracts (2% by volume of reaction mixture) were analyzed in this manner, taking the place of the unlabelled ligand and replacing the volume difference with buffer. In addition to the effluent extracts (PME and STP), the androgens 4-androsten-3,17-dione (AD) and 1,4-androsta-diene-3,17-dione (ADD) were also analyzed for androgen receptor binding potential due to their suspected involvement of causing gonopodium development in female mosquitofish (Jenkins *et al.*, 2001).

2.4 Aromatase activity assay

Aromatase is the endocrine enzyme that converts testosterone to 17-β-estradiol. Aromatase activity assays are involved with measuring the release of tritiated water from its reaction with [1β-^{3}H]androstenedione (Lephart and Simpson, 1991). Aromatase uses androstenedione as a substrate in its conversion to estrone (Figure 1.3). Aromatase inhibition would prevent the first step of this

reaction from occurring, thus stopping the formation of tritiated water when [1β-^{3}H]androstenedione is used as the substrate. Therefore, aromatase inhibition potential of test compounds can be determined by analyzing their ability to reduce the amount of tritiated water forming.

Protein enzyme (aromatase) was incubated along with tritiated steroid ([1β-^{3}H]androstenedione) and the test compound. The reaction mixture was measured by scintillation to determine the amount of tritiated water present after an incubation period. Based on the methods of Shilling *et al.*, (1999), an aromatase activity assay using rainbow trout egg that can show inhibition in a dose-response manner was used here. Ovarian follicles are believed to be the location of high aromatase activity due to the requirement of 17-β-estradiol in ovulation (Séralini and Moslemi, 2001).

2.4.1 Tissue preparation

Ovarian tissue from 3-year old sexually mature female rainbow trout were excised and preserved at –80°C. When ready for the next stage, the tissue was thawed, homogenized in 4:1 buffer-to-tissue ratio and pooled. The homogenate was then centrifuged at 11,000 g for 20 minutes. The resulting supernatant containing the microsomal fraction was collected and ultracentrifuged at 100,000 g for one hour. The microsomal pellet containing the aromatase enzyme from the ultracentrifugation was re-suspended in 0.5 mL of buffer and stored at –80°C until further use.

2.4.2 Assay procedure

The assay procedure was followed according to that described in Shilling *et al.*, (1999). Please refer to Figure 2.4 for a flowchart representation of this assay procedure. All reactions were carried out in triplicate in 5 mL borosilicate glass tubes. The reaction mixture contained 1 mM NADPH, 10 nM [1β-^{3}H]androstenedione, 10 mM $MgCl_2$ and 35 μL of the microsomal aromatase protein solution to a total volume of 500 μL. NADPH is required in excess as it is a cofactor in the reaction (see Figure 2.4 above). The aromatase inhibitor standard used here was 4α-hydroxyandrostenedione (HAD). A stock solution of 1 mg/mL of HAD was made in ethanol. Serial dilutions of the stock solution were made in ethanol and 5 μL additions were added to the reaction mixtures such that the inhibitor concentration range was 10-0.0001 μg/mL (logarithmic scale). Where effluent extracts were used, 5 μL additions of the extracts were added to the reaction mixtures. The reaction tubes were then incubated at 22°C for 90 minutes.

After incubation, 1 mL of chloroform was added to each tube to stop the reaction and the aqueous phase was diluted to 1.5 mL with distilled H_2O. The tubes were then centrifuged at 1000 g for 10 minutes. Following centrifugation, 1 mL of the aqueous phase from each tube was taken and placed into a new empty tube to which was added 1 mL of dextran-activated charcoal buffer A (1% charcoal, 0.1% dextran by weight). The new tubes were centrifuged at 2000 g for 15 minutes after which the supernatant was collected into separate 5 mL scintillation vials. Upon addition of 5 mL of scintillation cocktail to each tube (as

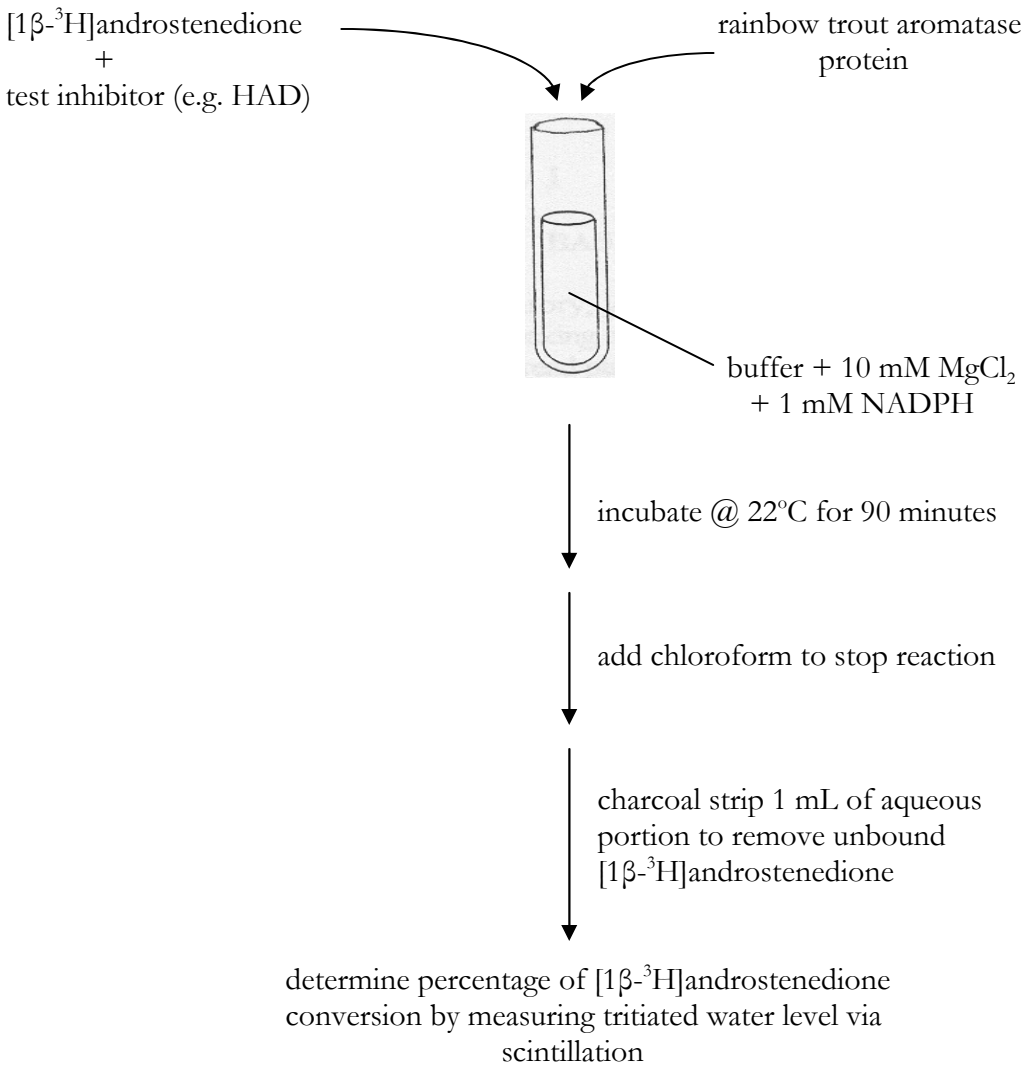

Figure 2.4: Flowchart diagram of rainbow trout aromatase activity assay

described in androgen receptor assay) the vials were measured by a scintillation counter. A blank set of tubes containing no inhibitor or effluent extract represented 100% activity (i.e. no inhibition) and all other counts were normalized to this value. Figure 2.5 shows the aromatase activity profile of increasing inhibitor standard (HAD) concentration.

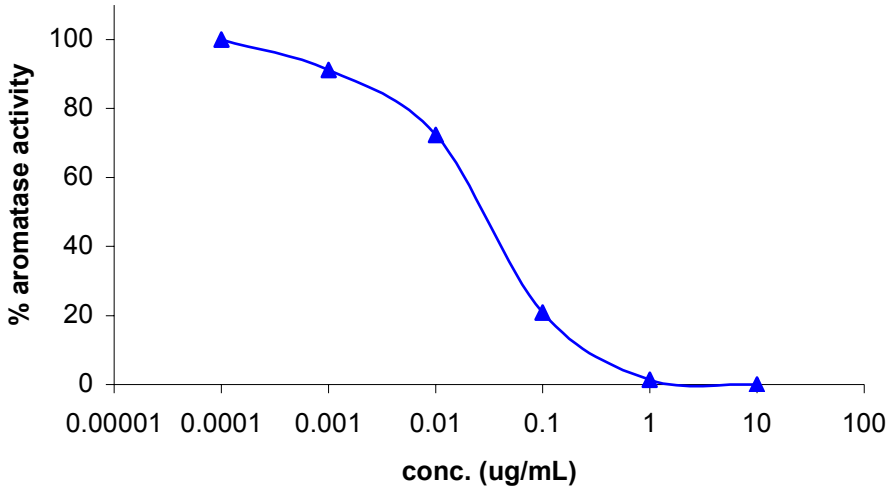

Figure 2.5: Relative aromatase activity in presence of increasing inhibitor (HAD) concentration

The log EC_{50} value of Figure 2.5 above was determined to be –2.511 by GraphPad Prism 3 software. This equates to a 7.18 nM EC_{50} concentration for HAD in the reaction mixture which contained 10 nM [1β-^{3}H]androstenedione. This agrees with other results of aromatase activity assays. HAD has been shown to be a strong inhibitor of aromatase in rainbow trout showing up to 80% inhibition at a concentration of 1.5 μM (Shilling *et al.*, 1999). Also, an IC_{50} value for HAD with aromatase (human placental microsome) using androstenedione as a substrate was determined to be 15 nM (Bullion *et al.*, 1990).

2.5 *In vitro* sex steroid analysis

The methods followed here were those found in McMaster *et al.*, (1995). Please refer to Figure 2.6 on the following page for a flowchart representation of

the *in vitro* steroid hormone assay. At the termination of exposure experiments, mosquitofish were sacrificed by an overdose of the fish anesthetic MS-222 and weighed to the nearest 0.001 g. Ovaries from each fish were then excised and placed in separate wells of a 24-well tissue culture plate. Each well contained 500 µL of Medium 199 (M199 containing Hank's salts without bicarbonate) adjusted to pH 7.2. Ovaries were carefully teased apart to release their eggs. The plates were then incubated for 18 hours at 20°C.

Following incubation, testosterone and 17-β-estradiol levels in the media were measured directly by radioimmunoassay (RIA). Standard curves for testosterone and 17-β-estradiol were made by creating a dilution series in 5 mL borosilicate glass tubes from 800 pg of respective steroid in 200 µL of Phosgel buffer (5.75 g Na_2HPO_4, 1.28 g $NaH_2PO_4*H_2O$, 1 g gelatin, 0.1 g Thimersol in 1 L of distilled H_2O adjusted to pH 7.6). The dilution range included 400, 200, 100, 50, 25, 12.5, 6.25, and 3.125 pg of steroid standard in 200 µL of Phosgel buffer. To these standards, 200 µL of radiolabelled steroid was added (^{3}H-testosterone or ^{3}H-17-β-estradiol). Then 200 µL of respective antibody solution was added (rabbit anti-testosterone/anti-17-β-estradiol). The reaction mixture was vortexed and incubated overnight at 4°C.

The following day, 200 µL of 1% activated charcoal-0.1% dextran Phosgel buffer was added to all tube and allowed to incubate for 10 minutes. When not in incubation or centrifugation, sample tubes were kept on ice at all times. The tubes were then centrifuged at 3000 g for 15 minutes and the supernatant collected in 5 mL scintillation vials. Scintillation cocktail (as described in section 2.3.2) was added to each vial and counts measured by a scintillation counter.

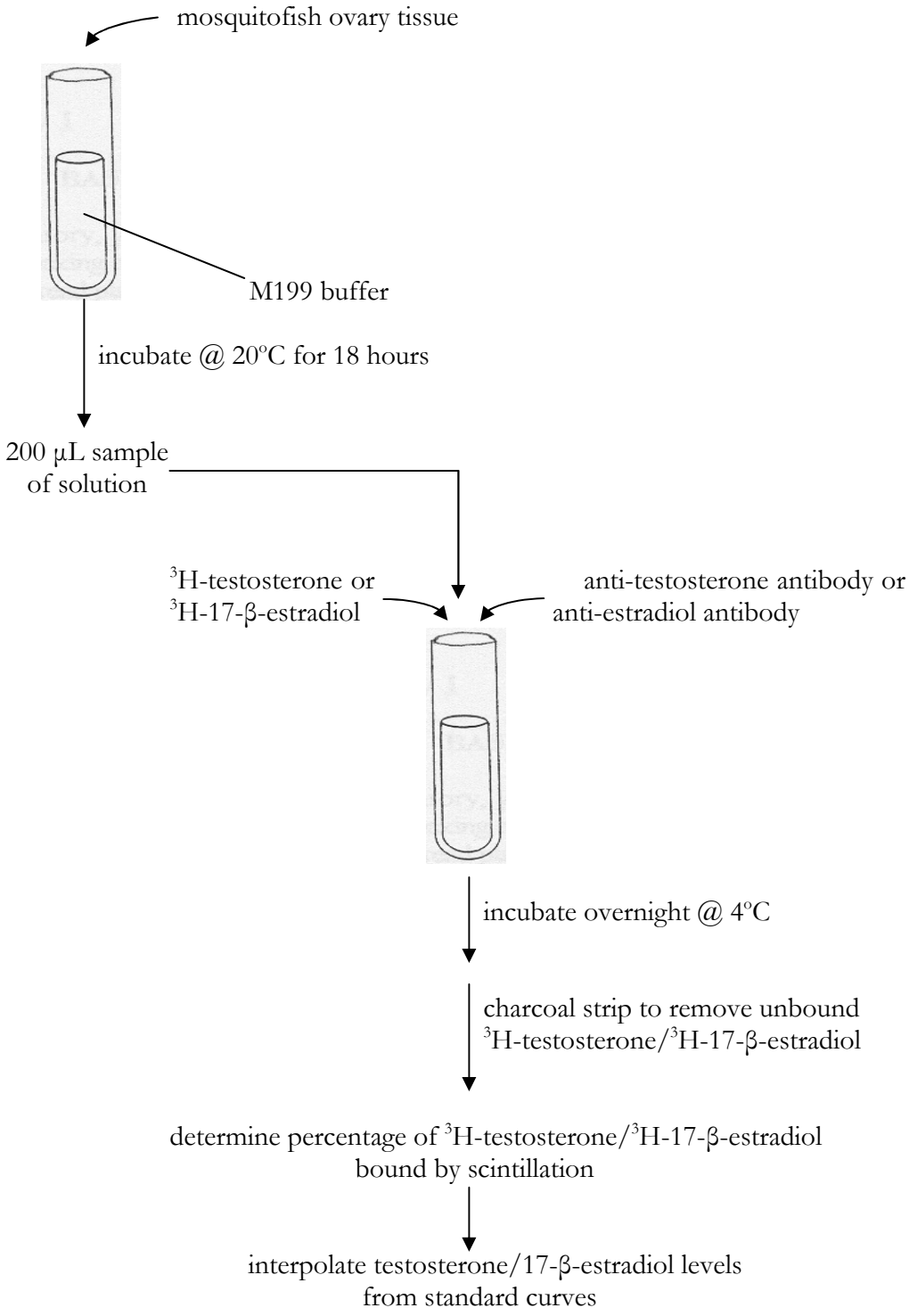

Figure 2.6: Flowchart diagram of the *in vitro* steroid assay performed using exposed female mosquitofish ovarian tissue

Similarly for mosquitofish exposure samples, 200 µL of media from each well was placed in reaction with 200 µL of respective antibody and 200 µL radiolabelled steroid hormone (separately for testosterone and 17-β-estradiol). Tubes were prepared and measured by scintillation as described above.

2.5.1 Statistical analysis

Steroid levels from mosquitofish ovary samples were interpolated from testosterone and 17-β-estradiol standard curves with a logistic function using GraphPad Prism 3 software. Steroid levels were then standardized according to total weight of each respective fish. Means for testosterone and 17-β-estradiol levels for each treatment (PME, STP, Tarawera river water, HAD and ethanol) were calculated along with standard error. A non-parametric Mann-Whitney unpaired test was performed to determine significant differences in respective steroid hormone levels between the PME and Tarawera river water treatments, as normal distribution for the data cannot be assumed. A non-parametric Kruskal-Wallis test was performed between the STP, HAD and ethanol treatments to determine statistical differences as three non-normally distributed means were compared in this case. The critical level of significance was assessed at $p < 0.05$ for all tests.

2.6 *In vivo* mosquitofish bioassay

Female mosquitofish were housed in 10 L aquaria under a 12:12 hour photoperiod. Temperatures were maintained between 26-28^{o}C and the aquaria were aerated with gentle air-streams. Treatments consisted of two replicates

(tanks A and B) of 10 fish each. Figure 2.7 shows a picture of the mosquitofish bioassay setup.

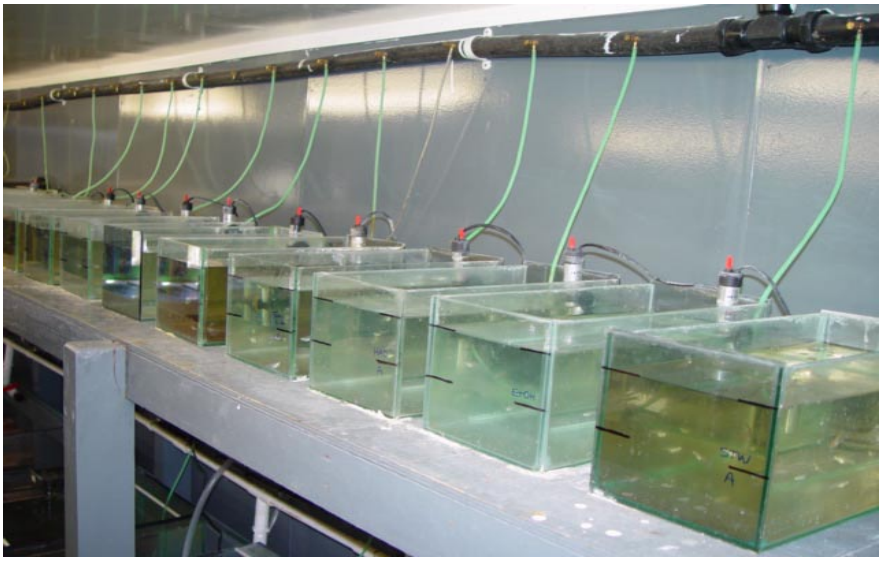

Figure 2.7: Mosquitofish bioassay setup

PME, STW and Tarawera river water treatments were at 50% dilution with dechlorinated tap water and all other treatments were with 100% dechlorinated tap water. A 50% dilution was chosen so as to facilitate the qualitative observations of the onset of potential anal fin ray fusion, which indicates the onset of anal fin ray elongation (Turner, 1941a). Having to pull fish out of their tanks to observe their anal fins would cause stress, which may alter sexual responses as seen with wild fish (McMaster *et al.*, 1995), and therefore may influence the potential onset of anal fin ray fusion. A delay in the onset of anal fin ray fusion in female mosquitofish is a qualitative indication of effluent androgenic potency. Also, gonopodial development in female mosquitofish exposed to this same New Zealand PME has been seen within 3 weeks at 15%

dilution (Ellis *et al.*, 2003). Microbial degradation of plant sterols normally occurs within 15-20 days, and this is the mechanism believed to be responsible for the androgenic responses seen in female mosquitofish from PME exposures (Bortone and Davis, 1994). The test volume of 10 L for each treatment underwent 50% static renewal daily.

The other treatments included methyl-testosterone (500 ng/L), AD (100 μg/L), ADD (100 μg/L, 10 μg/L, 1 μg/L), cyproterone acetate (250 μg/L, 25 μg/L, 2.5 μg/L) and HAD (100 μg/L). The methyl-testosterone concentration was chosen as it has been shown to be a minimal concentration required to produce anal fin ray elongation in female mosquitofish within 25 days (Turner, 1941b). AD has been shown to induce gonopodial development in female mosquitofish at a concentration of 10 mg/L in a laboratory setting (Husinger and Howell, 1994), and has been suggested to cause female mosquitofish masculinization at 40 ng/L in a Florida river (Jenkins *et al.*, 2001). So the concentration used here (100 μg/L) is chosen to show whether female mosquitofish masculinization could potentially occur at more environmentally relevant concentrations. ADD has not been investigated, so 10-fold dilutions were also used to potentially show a dose-response effect. The HAD (100 μg/L) and maximum cyproterone acetate (250 μg/L) concentrations were chosen as these were the highest possible concentrations to achieve in ethanol stock solutions due to their respective solubilities. All these substrates were made up in a stock using ethanol as a solvent. The concentrations of the respective stocks were appropriately made to achieve the final concentrations in the tanks stated above with 50 μL additions. One treatment having only 50 μL additions of neat

ethanol was also conducted as a negative control. Figure 2.8 and Table 2.2 summarize the mosquitofish bioassay design on the following page.

Methyl-testosterone is known as a potent androgen, which induces gonopodium development in female mosquitofish (Turner, 1941b) and was used as a positive control in these experiments. As mentioned previously, AD and ADD were investigated due to their implication in gonopodium development in female mosquitofish through microbial degradation of plant sterols found in PME (Jenkins *et al.*, 2001). Cyproterone acetate is a known mammalian anti-androgen that has been shown to be non-binding in rainbow trout androgen receptor, while at the same time binding to goldfish testes androgen receptor (Wells and Van Der Kraak, 2000). Its *in vivo* effects on gonopodial development in female mosquitofish were studied here with exposures of cyproterone acetate combined with methyl-testosterone. Although gonopodium development in female mosquitofish due to aromatase inhibition has been implied not to occur (Orlando *et al.*, 2002), direct *in vivo* evidence was lacking. Here HAD was investigated for its potential to induce gonopodium development in female mosquitofish. All treatments lasted 25 days with daily observational accounting of fin ray elongation and fusion. At the termination of the exposure the fish were sacrificed by overdose of the fish anesthetic MS-222, the ovaries of each fish excised and used for *in vitro* steroidogenesis analysis (described in section 2.4) and digital photos of their anal fins were taken using a confocal microscope with an attached digital camera. The anal fin ray 4:6 ratio was then determined for each fish in each treatment using Image Tool software.

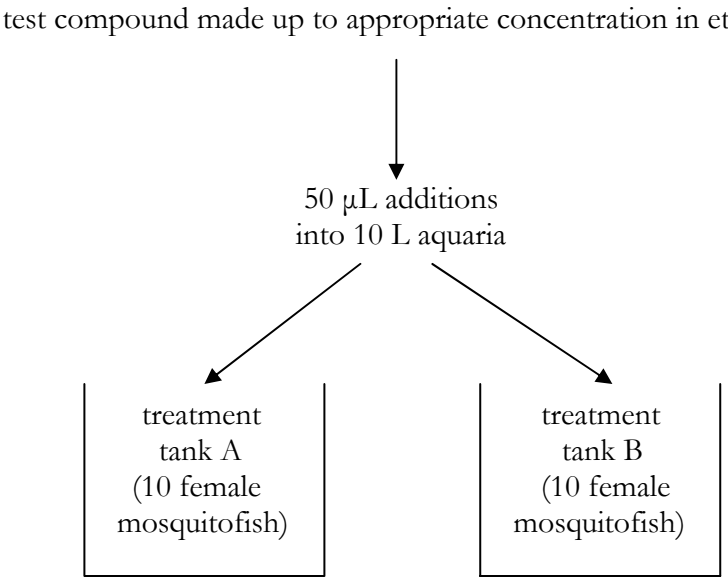

Figure 2.8: Mosquitofish bioassay design. All treatments were performed in duplicate (tank A and B) and under 50% daily static renewal.

treatment name	**final concentration in tanks A and B (µg/L)**
AD	100
ADD 100	100
ADD 10	10
ADD 1	1
CA 250	250
CA 25	25
CA 2.5	2.5
MT	0.5
HAD	100

Table 2.2: Treatments used in mosquitofish bioassay and their respective concentrations. CA represents cyproterone acetate, MT represents methyl-testosterone. All CA treatments had 50 µL MT additions as well. Effluent (PME, STP) and upstream New Zealand reference concentrations were at 50% dilution.

2.6.1 Statistical analysis

As reported in Angus *et al.*, (2001), anal fin ray ratios for mosquitofish follow a normal distribution when log-transformed. Thus, all fin ray ratios were log-transformed prior to ANOVA analysis using GraphPad Prism 3 software, as we were comparing more than two means in all cases. The critical level for significance was assessed at $p < 0.05$. A Dunnett's post-hoc test was performed for all graphs to elucidate specific significant differences where detected, as we were interested in comparing all fin ray ratios from the various treatments to the negative control mosquitofish exposure (EtOH). The only exception was for the cyproterone acetate treatment data (Figure 3.16), where we wanted to compare all the columns between themselves and thus used a Bonferroni's post-hoc test.

3.0 RESULTS AND DISCUSSION

3.1 Androgen receptor assay

Competitive binding curves are useful tools in that they show the binding of test compounds as a sigmoidal dose-response curve, which is amenable to the calculation of an EC_{50}. An EC_{50} is a toxicological term used in this case to describe the concentration of compound that elicits 50% binding to the receptor. The competitive binding curves in terms of ^{3}H-testosterone (tritiated-T) displacement for testosterone, AD, ADD and 17-β-estradiol are shown in Figure 3.1 and Figure 3.2 respectively. Their respective EC_{50} values were determined by GraphPad Prism 3 software and are tabulated in Table 3.1, except for 17-β-estradiol, which did not bind to the androgen receptor (Figure 3.2). Relative binding affinities (RBAs) were another way to show the potency of compounds to bind to steroid receptors. They are defined as the ratio of the EC_{50} of androgen standard (testosterone in this case) to the EC_{50} of the test compounds, multiplied by 100. Thus, the RBA of testosterone was defined to be 100%.

The RBAs of AD (241%) and ADD (164%) indicate that they were relatively potent binders to the rainbow trout brain androgen receptor. This means that a relatively smaller amount of AD and ADD can potentially elicit the same androgenic response as a relatively higher amount of testosterone. The AD result here (241%) agrees with another study where AD is shown to be more potent than testosterone (187.6%), also in rainbow trout brain androgen receptor (Wells and Van Der Kraak, 2000). However, AD was found to be non-binding in Atlantic croaker brain androgen receptor and showed only very weak binding (1.6%) to Atlantic croaker ovary androgen receptor (Sperry and Thomas, 1999).

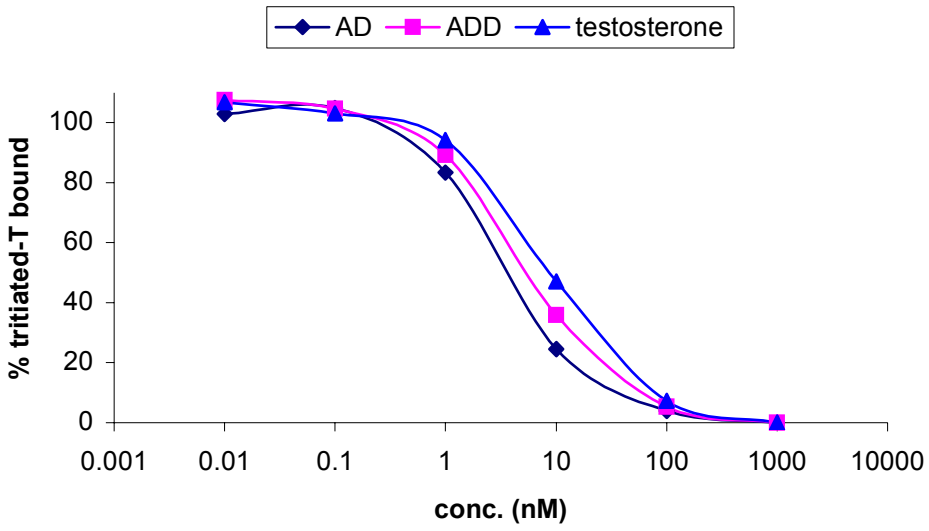

Figure 3.1: Rainbow trout androgen receptor competitive binding curves for testosterone, AD, ADD

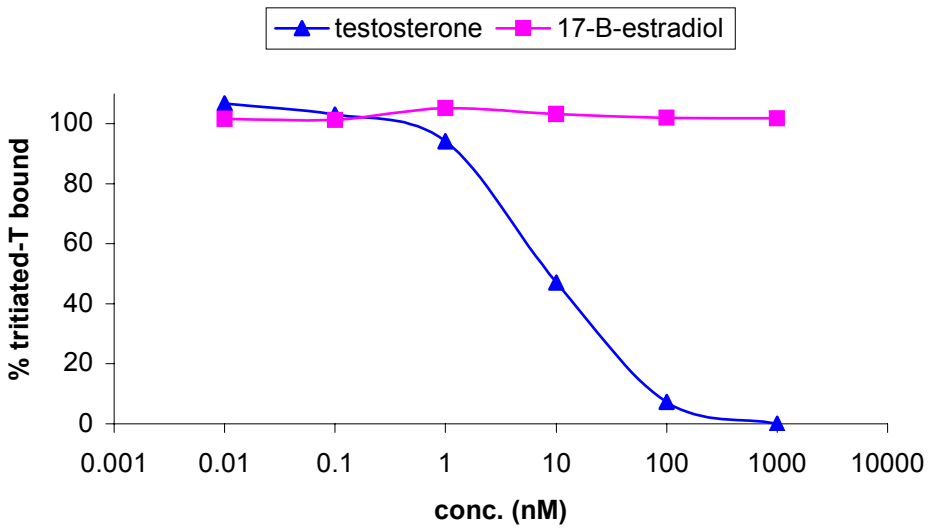

Figure 3.2: Rainbow trout androgen receptor competitive binding curves for testosterone and 17-β-estradiol

Steroid	EC_{50} (nM)	RBA (%)
Testosterone	8.2	100
AD	3.4	241
ADD	5.0	164

Table 3.1: EC_{50} values and relative binding affinities (RBA) for testosterone, AD, ADD as determined from their competitive binding curves

AD binding in goldfish testes (52.6%), goldfish brain (57.1%) and goldfish ovaries (67.9%) have also been reported (Wells and Van Der Kraak, 2000). These results indicate that androgen receptors from different species have different affinities for the same androgen, which is an important consideration if an androgen receptor binding assay is being used to detect the presence of a potential androgenic xenobiotic. Moreover, appreciable binding of 17-β-estradiol to goldfish testes androgen receptor (16.3%) reported by Wells and Van Der Kraak (2000) show that the selection of species tissue for androgen receptor source may affect the selectivity of the assay to screen for androgens.

Another important result here is the relatively strong potency of ADD to rainbow trout androgen receptor (164%). This is the first reported EC_{50} value for ADD using fish androgen receptor. ADD is reported to be of higher yield (30%) than AD (13%) from conversion of β-sitosterol, a common plant sterol found in PME, with *M. smegmatis* (Mahato and Garai, 1997). While there have been debates about the presence of AD in waters receiving PME discharges, due to the microbial conversion of plant sterols in the PME (Jenkins *et al.*, 2001; Durhan *et al.*, 2002), the potential prevalence of ADD has not been examined. Androgenic responses due to the presence of ADD in PME appear to be a possibility.

The rainbow trout androgen receptor assay was then used to determine the relative androgenic potencies of two PMEs and a STP. Fish androgen receptor assays have been used to determine the relative potencies of PMEs in the past (Hewitt *et al.*, 2000; Ellis *et al.*, 2003) but these studies used androgen receptor isolated from goldfish testes which, as mentioned earlier, have been shown to have moderate affinity for 17-β-estradiol (Wells and Van Der Kraak, 2000). Rainbow trout brain AR, as seen from Figures 3.1 and 3.2, has a very specific affinity for androgens. Below are the competitive binding curves for the New Zealand PME and its upstream reference sample (Figure 3.3), Canadian PME and its upstream reference sample (Figure 3.4), and the New Zealand STP with a reference blank sample (Figure 3.5).

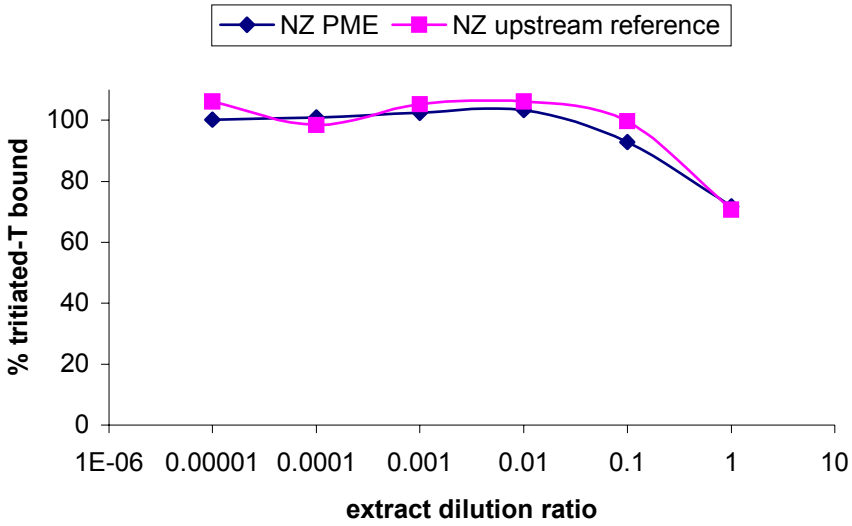

Figure 3.3: Competitive binding curve of the New Zealand PME sample extract and its upstream reference sample extract

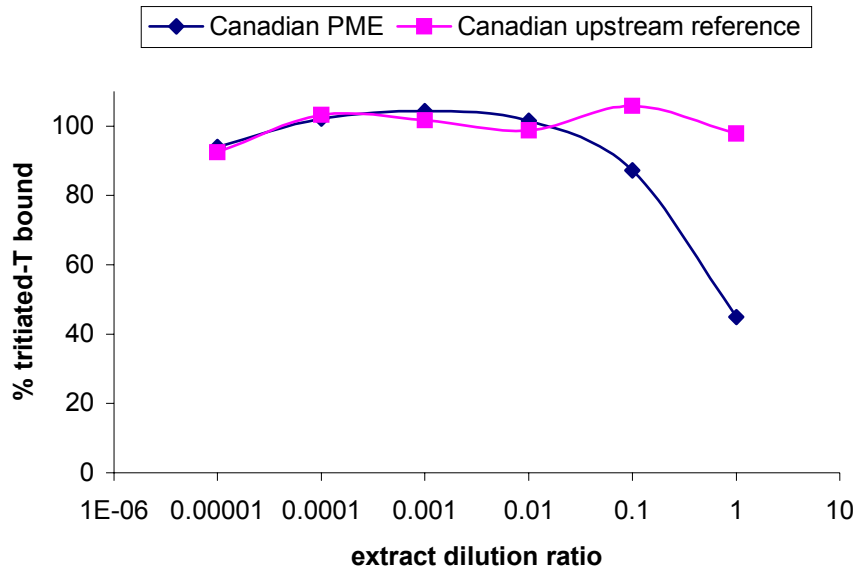

Figure 3.4: Competitive binding curve of the Canadian PME sample extract and its upstream reference sample extract

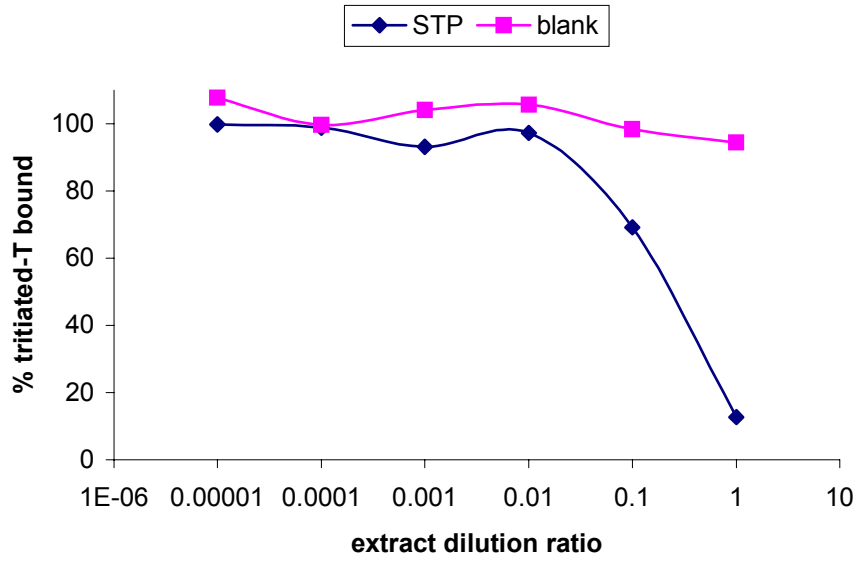

Figure 3.5: Competitive binding curve of the New Zealand STP sample extract and its reference blank sample extract

Since the competitive binding studies with the effluent extracts dealt with an unknown starting concentration of potential androgenic xenobiotics, the binding to the androgen receptor is not measured relative to concentration, but rather to the dilution factor of the extract sample. This was done in order to potentially to produce the sigmoidal dose-response curve for the effluent extracts and establish an EC_{50} that could be related arbitrarily to testosterone EC_{50} and establish a "testosterone-equivalent" value. However, the curves are not complete and determination of the EC_{50} is not possible.

While the New Zealand PME extract appears to be relatively non-binding (Figure 3.3), the Canadian PME (Figure 3.4) and New Zealand STP (Figure 3.5) show a trend toward increasing androgen receptor binding. The Canadian PME was screened for the presence of testosterone, AD and ADD; and these were found to be non detectable (detection limit of 1 ng/mL). Also, the Canadian PME has failed to show significant gonopodial development in female mosquitofish (Hardy, 2002), which suggests that androgens are not present at high enough concentration to elicit this response. It may possibly be detecting the presence of androgens that are too low in concentration to cause this *in vivo* effect, or be detected by GC-MS. The androgen receptor binding seen from the STP extract may be due to testosterone excreted from humans. While it is known that humans excrete relatively large amounts (0.002-0.10 mg per day) of 17-β-estradiol (Huang and Sedlak, 2001), and the presence of other endogenous and synthetic estrogens have been detected in STP (Purdom *et al.*, 1994), relatively little work has been done on the detection of androgens in STP. Figure 3.5 shows that androgenic compounds in STP may be approaching detectable levels.

3.2 Aromatase activity assay

The aromatase activity curve for the aromatase inhibitor standard HAD has already been shown in Figure 2.4 and its EC_{50} has been determined to be 7.18 nM. The aromatase activity curves for the New Zealand PME and its upstream reference sample (Figure 3.6), the Canadian PME and its upstream reference sample (Figure 3.7), and the New Zealand STP and a reference blank sample (Figure 3.8) are shown. While the curves are again incomplete, trends toward increasing aromatase inhibtion can be seen with in the Canadian PME (Figure 3.7) and the STP (Figure 3.8), with a slight trend noticed with the New Zealand PME (Figure 3.6). The effluent extracts were screened for the presence of HAD using GC-MS and none was found, nor was it expected to be found.

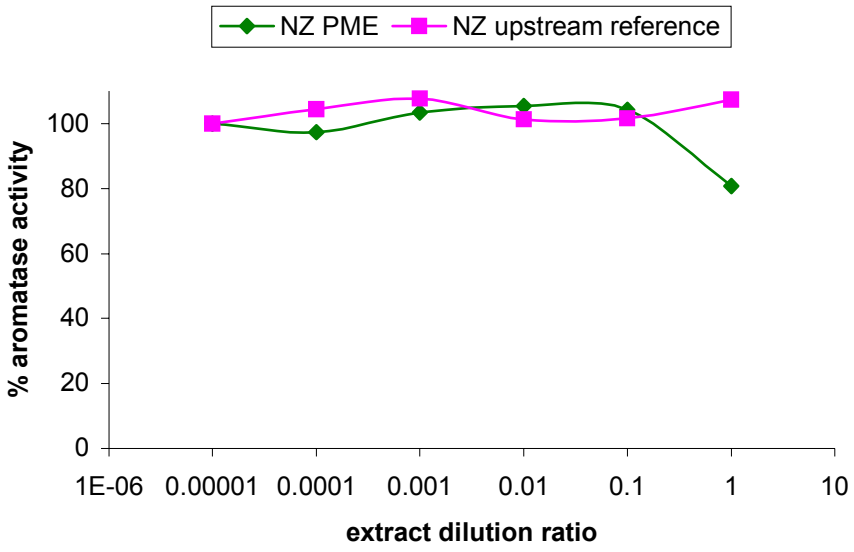

Figure 3.6: Aromatase activity curve of the New Zealand PME sample extract and its upstream reference sample extract

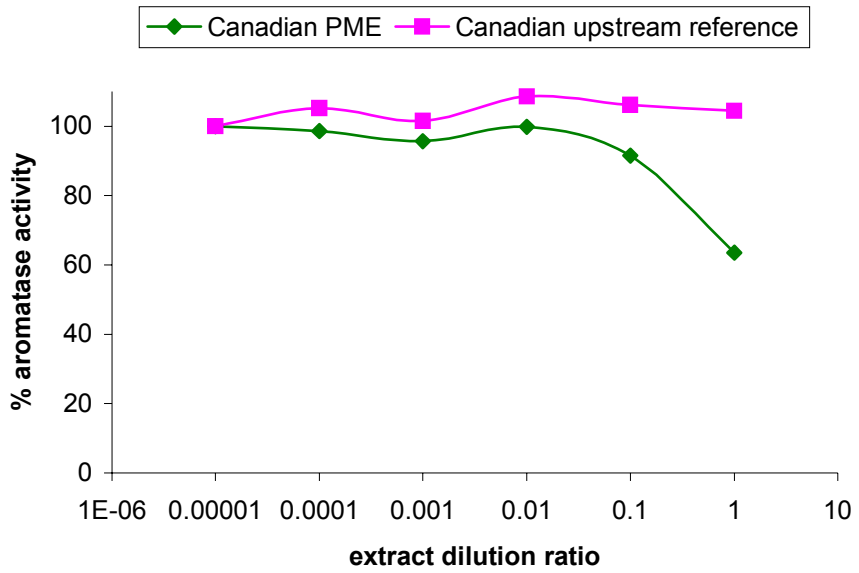

Figure 3.7: Aromatase activity curve of the Canadian PME sample extract and its upstream reference sample extract

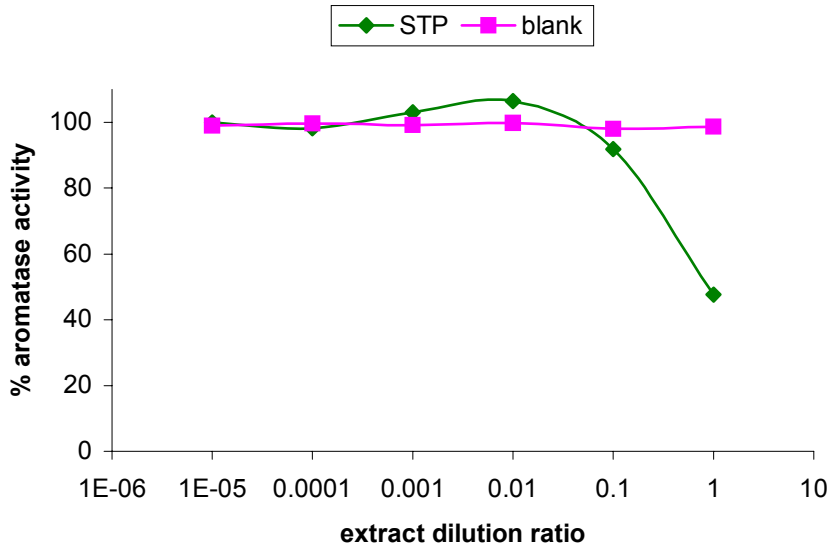

Figure 3.8: Aromatase activity curve of the New Zealand STP sample extract and its blank reference sample extract

3.3 *In vitro* mosquitofish ovary sex steroid levels

The results from the *in vitro* testosterone analysis of the New Zealand PME and upstream reference treated female mosquitofish are shown in Figure 3.9. The ovarian testosterone levels for the PME treated female mosquitofish are significantly lower than the upstream reference treated fish (Mann-Whitney U test, $p < 0.05$). While this may suggest the presence of androgenic compounds in the PME that are feeding back in the HPG axis (Figure 1.1) to stop the production of testosterone, our rainbow trout androgen receptor binding assay showed no difference in binding between the New Zealand PME extract and its upstream reference (Figure 3.3). The decreased production of testosterone in the PME exposed female mosquitofish may be due to non-steroidal compounds present in the effluent. Compounds causing reduced testosterone levels in mummichog have been isolated from a Canadian PME, but their identities still remain unknown (Hewitt *et al.*, 2002). Also, as already mentioned in section 3.1, androgen receptors from different fish species have different affinities for the same androgen. Thus, another possibility may be that the mosquitofish androgen receptors are more sensitive to the xenobiotic androgens that may potentially be present in the PME than rainbow trout brain androgen receptors.

As seen in Figure 3.10, ovarian testosterone levels in STP treated female mosquitofish are lower than the ethanol-treated negative control (EtOH) mosquitofish, while the aromatase inhibitor HAD, as expected, was found not to alter testosterone levels. Statistical analysis of the testosterone levels shown in

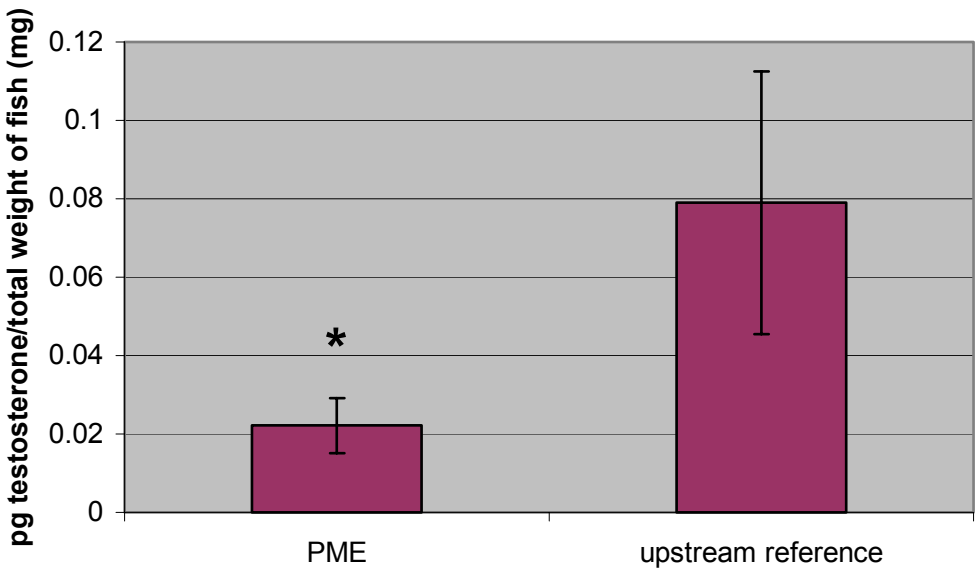

Figure 3.9: Testosterone levels in female mosquitofish ovaries exposed to New Zealand PME and upstream reference. Asterisk denotes a significant difference between the means (Mann-Whitney U test, $p < 0.05$).

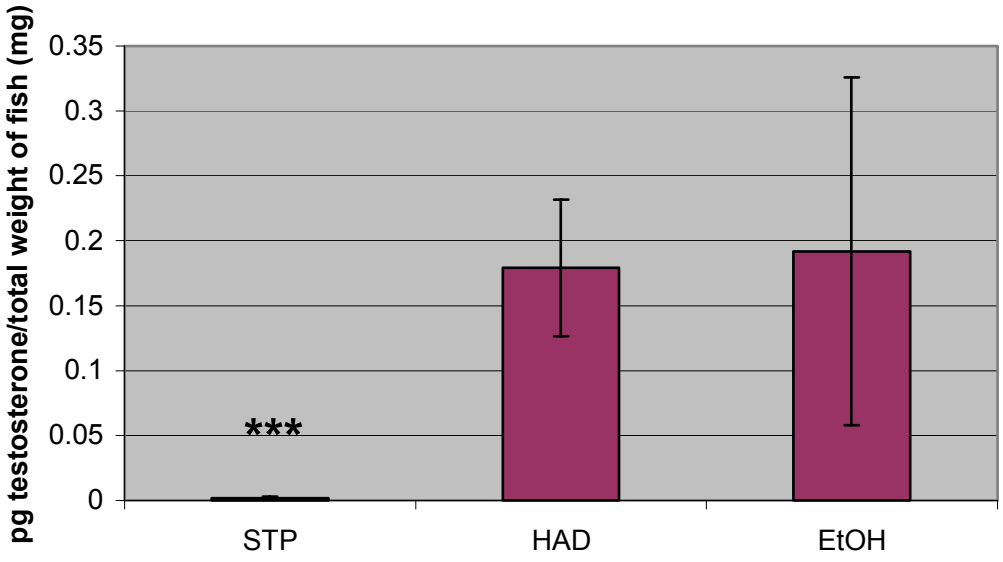

Figure 3.10: Testosterone levels in female mosquitofish ovaries exposed to STP, HAD, and EtOH (negative control). Asterisks for the STP treatment denote a significant difference from the HAD treatment (Kruskal-Wallis test, $p < 0.001$).

Figure 3.10 only shows a significant difference between the STP and HAD treatments (Kruskal-Wallis test, $p < 0.001$. Nevertheless, STP showed some degree of binding to the rainbow trout androgen receptor (Figure 3.5) indicating the low presence of androgens in the effluent, which may also be causing the decreased testosterone production seen here through negative feedback in the HPG axis. While detection of androgenic compounds in STP are not widely reported, another study has found decreased circulating testosterone levels in female carp caught downstream from a STP discharge (Folmar *et al.*, 1996).

Ovarian 17-β-estradiol levels for PME and upstream reference treated female mosquitofish are shown in Figure 3.11. There is no significant difference in the 17-β-estradiol levels (Mann-Whitney U test, 95% confidence), which suggests that testosterone levels should also not be different. This is contradicted by our results shown in Figure 3.9, and may be due to the release of 17-β-estradiol that has been stored in the follicles. However, it may also be due to the presence of AD in order to maintain 17-β-estradiol levels (see Figure 1.3 for gonadal steroidogenesis pathway). AD can be formed by microbial conversion of β-sitosterol, which is commonly found in PME (Denton *et al.*, 1985), and has been detected in treated effluent (165.4 μg/L) and treated effluent extracts (147.8 μg/L) from this particular mill previously (van den Heuvel *et al.*, 2003). GC-MS analysis of the New Zealand PME extract found no presence of AD. Therefore, if AD is present in the New Zealand PME sample, it is at a level undetectable by the rainbow trout androgen receptor assay.

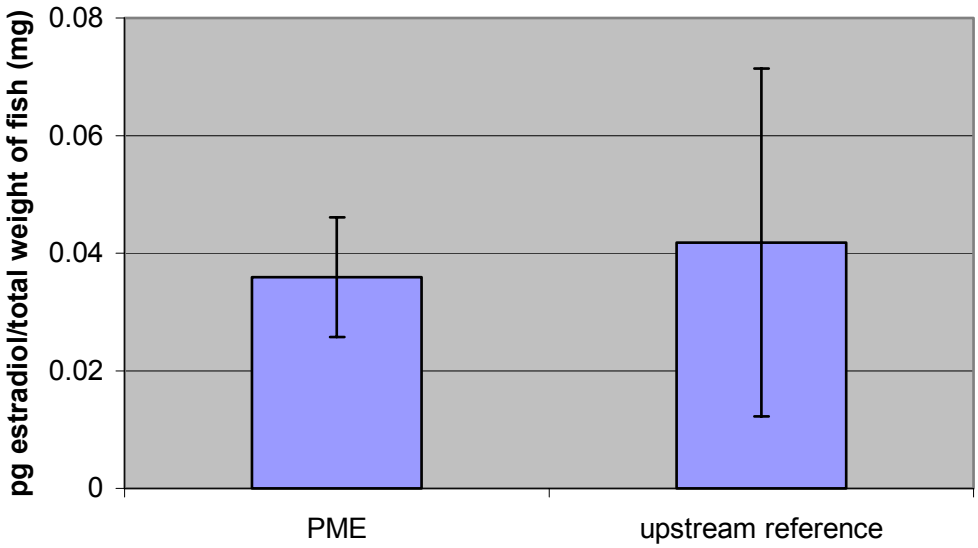

Figure 3.11: 17-β-estradiol levels in female mosquitofish ovaries exposed to New Zealand PME and upstream reference. No significant difference between the means was detected (Mann-Whitney U test, 95% C.I.).

Figure 3.12 shows the ovarian 17-β-estradiol levels in the STP, HAD, and EtOH treated female mosquitofish. The large error associated with the EtOH treatment does not allow for any statistical difference to been seen between the treatments (Kruskal-Wallis test, 95% confidence), but noticeable trends can still be observed. 17-β-estradiol levels for the aromatase inhibitor HAD are lower than the negative control EtOH, as expected, due to its blockage of testosterone conversion to 17-β-estradiol. The STP treatment also showed lower 17-β-estradiol levels compared to the negative EtOH control. This may be attributed to the aromatase inhibiting potential of the effluent from a combination of both steroidal androgenic compounds, as suggested in section 3.1 (Figure 3.5), and non-steroidal aromatase inhibiting compounds (section 3.2, Figure 3.8), which

may be present. Currently, there are no studies available that have thoroughly investigated the aromatase inhibiting potential of STP or PME.

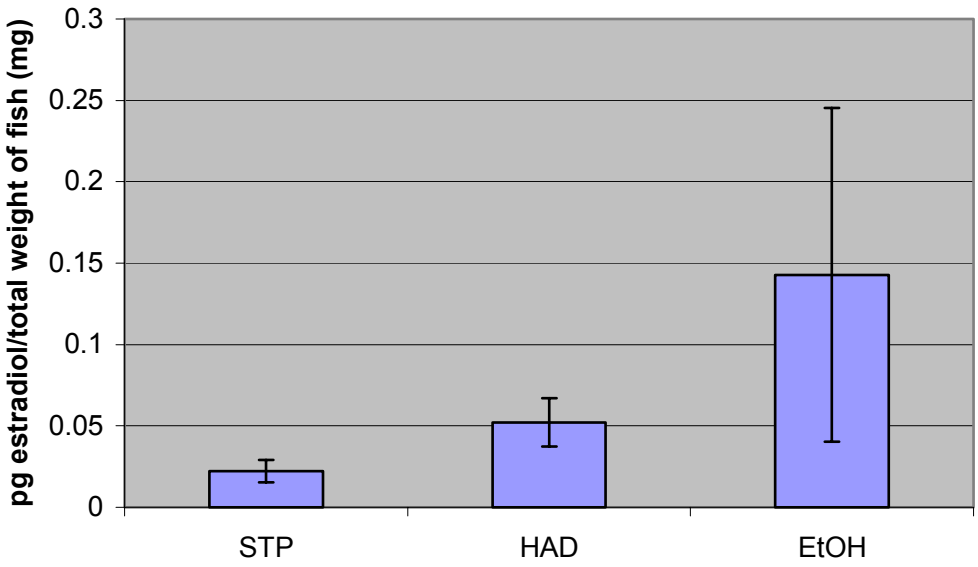

Figure 3.12: 17-β-estradiol levels in female mosquitofish ovaries exposed to STP, HAD, and EtOH (negative control). No significant differences between the means were detected (Kruskal-Wallis test, 95% confidence).

3.4 Mosquitofish bioassay

The 4:6 anal fin ray ratios for the MT, EtOH, STP, New Zealand PME and the upstream reference treated female mosquitofish are charted in Figure 3.13. One-way ANOVA analysis (95% confidence interval) showed a significant difference in the variances ($p < 0.0001$). A Dunnet post-hoc test, having EtOH as the control, found no significant difference between the STP, PME or upstream reference treatments. The MT treatment, used a positive control, was significantly different ($p < 0.01$). Inter-tank differences (i.e. between tanks A and B for the same treatment) using Bonferroni's post-hoc test, which allows for

selection of any two treatments, were not found. The New Zealand PME and STP do not appear to induce gonopodial development in this bioassay. From the previous sections (3.1-3.3), we have seen evidence suggesting the presence of androgens at very low levels in the New Zealand PME and STP effluent samples. Therefore, if they are truly present, they must be at levels low enough not to cause anal fin ray elongation in female mosquitofish in this bioassay. Lower dilutions of effluent and/or longer bioassay duration might yield different results. Inter-species (mosquitofish vs rainbow trout) differences in sensitivity to androgens may also, in part, account for the lack of *in vivo* androgenic response in the mosquitofish seen in Figure 3.13.

Interestingly, Ellis *et al.*, (2003) found gonopodial development in female mosquitofish exposed to PME samples from the same mill used in this study. Ellis *et al.*, (2003) also found appreciable binding in the goldfish testes androgen receptor assay with solid-phase extracts of the effluent. The PME samples were taken in 1999 and since then, there have been reported changes in the effluent quality (van den Heuvel *et al.*, 2003). Between 1999 and 2003 there has been a gradual decrease in the total suspended solids of the effluent (van den Heuvel *et al.*, 2003). This could potentially be reflected in the results shown in Figure 3.13 as low molecular weight compounds are highly adsorbed to suspended particulate matter. Also noted in van den Heuvel *et al.*, (2003) is the change in specific resin acid ratios in the effluent between 1999 and 2003, which may indicate a change in the microbial ecology of the treatment system. For the Canadian PME, Hardy (2002) found no visible evidence of anal fin elongation in female mosquitofish exposed for 20 weeks using a flow-through setup. Total sterol concentrations for

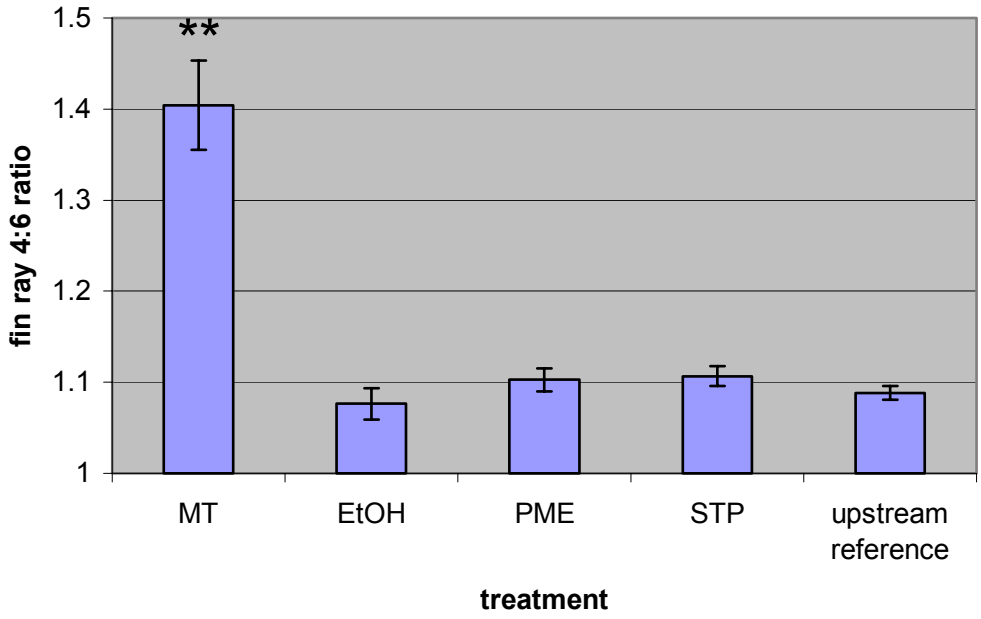

Figure 3.13: 4:6 anal fin ray ratios for female mosquitofish exposed to effluents (PME, STP) and upstream reference from New Zealand at 50% dilution, and controls (MT at 500 ng/L, EtOH). One-way ANOVA analysis with Dunnet's post-hoc test show MT significantly different from EtOH ($p < 0.01$).

the Canadian treated effluent ranged from 46 to 298 µg/L, which is comparable to the total sterol concentration of the New Zealand treated effluent (288 µg/L). This also suggests that there is a lack of microbial conversion of plant sterols to steroids, which may possibly be due to the structure of the microbial ecology of the effluent treatment system.

Treatment of female mosquitofish with 100 µg/L of AD and 100 µg/L of ADD yielded significant induction of anal fin elongation (Dunnet's post hoc test, $p < 0.01$) in relation to the EtOH control treatment, whereas the 100 µg/L HAD treatment did not, as seen in Figure 3.14. This suggests, in agreement with Orlando *et al.*, (2002), that aromatase inhibition does not induce gonopodial

development in female mosquitofish. In Figure 3.14, we also see a higher induction of anal fin ray elongation from ADD treatment than AD treatment. The dose-response assessment of ADD treatment can be seen in Figure 3.15. It is shown that possible masculinization effects of ADD in female mosquitofish can only be significant if concentrations are in the 100 μg/L range. Along with the fact that ADD appears to be a more potent inducer of anal fin ray elongation (Figure 3.14), suggestions that AD may be inducing masculine effects in the wild at concentrations of 40 ng/L (Jenkins *et al.*, 2001) are not supported here. Furthermore, Durhan *et al.*, (2002) have shown that androgenic extracts from the same sample site (Fenholloway River, Florida) do not contain AD. They do not suggest what compounds may possibly be exerting the androgenic response. Even considering that ADD is converted in higher yields than AD from β-sitosterol by *M. smegmati*s (Mahato and Garai, 1997), ADD is unlikely to be responsible for these effects. Although ADD was not detected by GC-MS in this New Zealand PME sample extract however, further investigation into this possibility for other samples may be warranted.

Figure 3.16 shows the effect of the mammalian non-steroidal anti-androgen cyproterone acetate (CA) on gonopodial development in female mosquitofish. The CA treatments were in conjunction with the positive control (MT) additions. The results in Figure 3.16 show a significant reduction in gonopodial development in female mosquitofish exposed to CA at a concentration of 250 μg/L (Bonferroni's post hoc test, $p < 0.05$). This suggests that while CA has reported to be non-binding in rainbow trout (Wells and Van Der Kraak, 2000) and Atlantic croaker (Sperry and Thomas, 1999), it apparently is

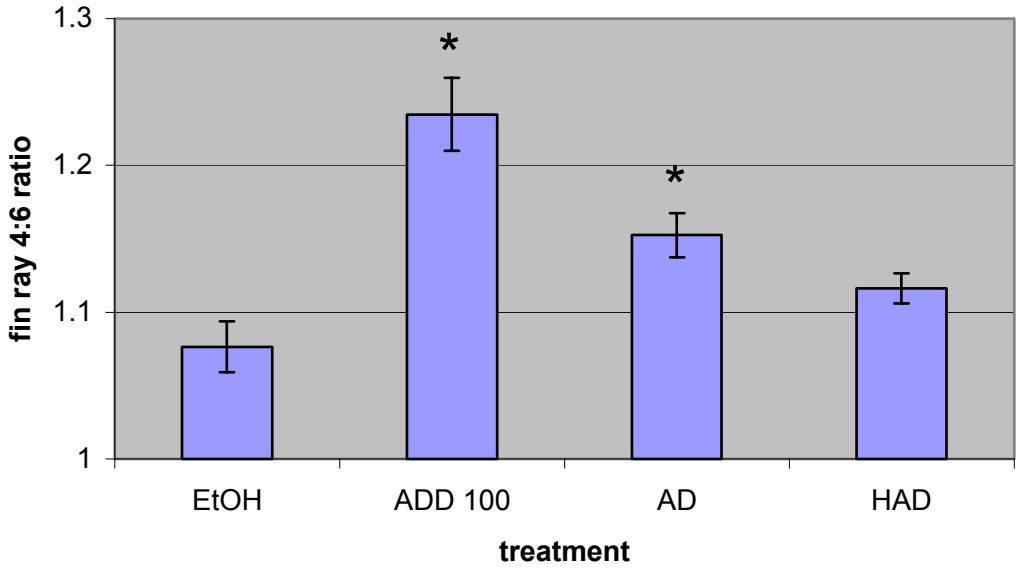

Figure 3.14: 4:6 anal fin ray ratios for female mosquitofish exposed to ADD, AD, and HAD, all at 100 µg/L, along with negative control (EtOH). One-way ANOVA analysis with Dunnet's post-hoc test show ADD and AD to be significantly different from EtOH ($p < 0.05$).

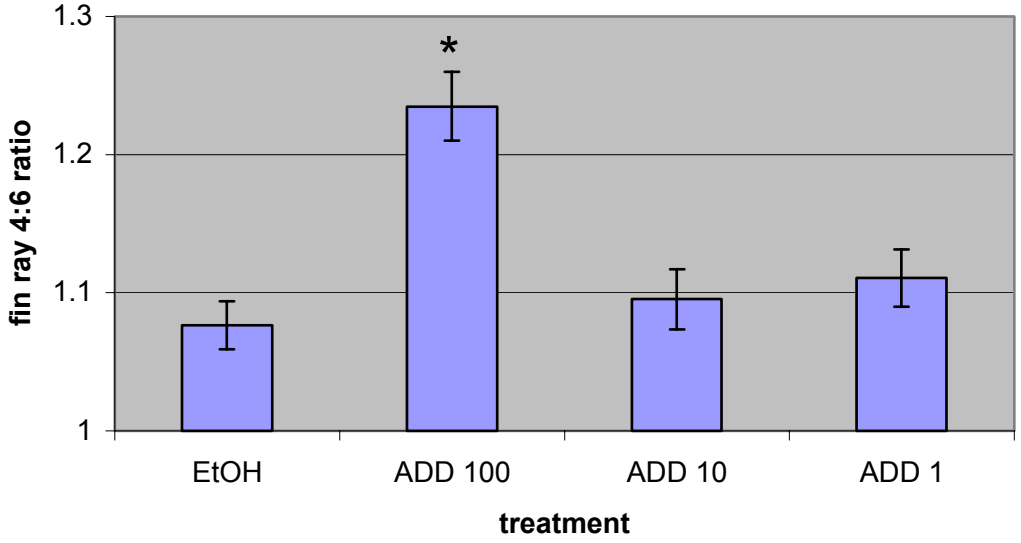

Figure 3.15: Dose-response of ADD to anal fin ray elongation in female mosquitofish. Treatment concentrations are 100, 10 and 1 µg/L for ADD 100, ADD 10 and ADD 1 respectively. One-way ANOVA analysis with Dunnet's post-hoc test show ADD treatment at 100 µg/L to be significantly different from EtOH ($p < 0.05$).

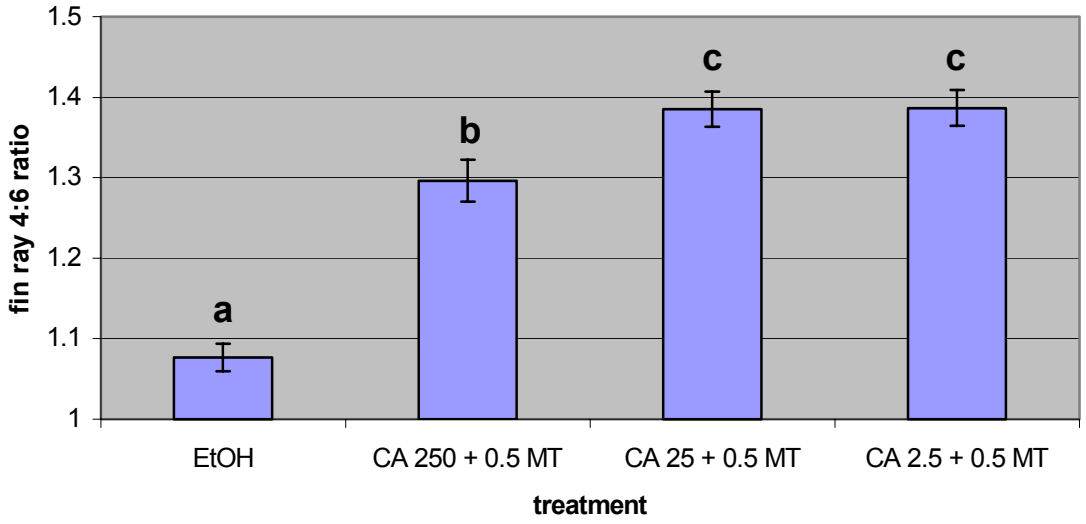

Figure 3.16: 4:6 anal fin ray ratios for female mosquitofish exposed to 50 µg/L MT and increasing concentrations of cyproterone acetate (CA). CA 250, CA 25 and CA 2.5 denote concentrations of 250, 25, and 2.5 µg/L respectively. One-way ANOVA analysis with Bonferroni's post-hoc test was performed. Treatments with same letter are not significantly different from each other.

binding to mosquitofish androgen receptors, causing a decrease in the androgenic effect of MT by competition. This again illustrates inter-species variability, and therefore must be a consideration in designing *in vitro* tests for detection of steroidal compounds. Also, the time required to first visible signs of anal fin elongation were observed to be delayed with increasing CA concentration. Normally, with the positive control anal fin elongation occurs with 5-7 days. In the 2.5 µg/L CA treatment, this was not delayed. For the 25 µg/L CA treatment, elongation was delayed by 7 days and for the 250 µg/L treatment it was delayed by 14 days. Once initiated, elongation of anal fin rays 4, 5 and 6 should occur rapidly (Turner, 1941b). This supports the theory that although anti-androgens may not decrease overall androgenic responses in organisms, they may affect the

timing of these responses, which would have a critical affect depending on the organisms' stage of development (IPCS, 2002). Effluents could be tested for their anti-androgenic potential by testing whether their extracts significantly reduce or delay the onset of gonopodial development in female mosquitofish treated with a known androgen, in a bioassay design as described in this study. To date, there are no studies on the anti-androgenic potential of effluents available.

4.0 CONCLUSIONS AND RECOMMENDATIONS

Addressing the hypotheses stated in the research objectives (section 1.7):

1) The results from this study show the potential of the Canadian PME and New Zealand STP to elicit *in vitro* androgenic responses in the rainbow trout androgen receptor assay. The *in vitro* aromatase activity assay results suggest aromatase inhibition for all the effluent samples, with the gonadal sex steroid analysis results further supporting this for the New Zealand PME and STP. However, the *in vivo* mosquitofish bioassay results do not show any evidence of a whole organism androgenic response. *In vivo* responses may manifest if lower dilutions and longer durations were to be used for the mosquitofish bioassay. Also, a flow-through setup for the bioassay would be an improvement over the static-renewal procedure, as steroidal compounds tend to have limited availability to the mosquitofish due to the relatively low solubilities in water.

2) At the concentrations used in this study, STP appears to have a stronger *in vitro* response than the New Zealand PME and Canadian PME. The STP shows stronger binding in the rainbow trout androgen receptor assay and stronger inhibition in the aromatase activity assay than either PME. STP also showed stronger suppression of testosterone and 17-β-estradiol production in exposed mosquitofish than the New Zealand PME. PME has been reported to induce male responses in eelpout in the wild (Larsson *et al.*, 2000) and in whole organism laboratory studies using mosquitofish (Ellis *et al.*, 2003). STP has been studied for its ability to induce estrogenic effects due to the presence of estrogenic compounds (Sumpter, 1995), but its potential androgenic effects has not been examined. Very recent work on the New Zealand STP effluent extract

sample has detected the presence of AD and ADD in addition to estrogens (van den Heuvel, pers. comm.). Further work on the androgenic potential of STP is warranted.

3) The results from this study suggest that different fish species have different sensitivities to androgens and anti-androgens. Rainbow trout brain androgen receptor was found to have a very strong affinity for AD and ADD, whereas goldfish testes androgen receptor has a medium affinity for AD (Wells and Van Der Kraak, 2000) and Atlantic croaker has a very low affinity at all for AD (Sperry and Thomas, 1999). Furthermore, goldfish testes has shown to have appreciable affinity for 17-β-estradiol (Wells and Van Der Kraak, 2000). The results from this study also suggest that mosquitofish androgen receptors have an affinity for the mammalian anti-androgen cyproterone acetate, while rainbow trout androgen receptor has shown to have no affinity for it (Wells and Van Der Kraak, 2000). Thus, inter-species variability is a factor when considering *in vitro* tools to detect androgenic compounds in effluents. The rainbow trout brain androgen receptor assay has shown to be very sensitive and specific for androgens and should therefore be considered as a tool for trying to isolate and identify xenobiotic androgens in effluents. This study could also be repeated using mosquitofish gonadal tissue for the *in vitro* tests.

4) The *in vitro* tests suggest an androgenic response potential for the Canadian PME and New Zealand STP, but the *in vivo* results for the New Zealand STP here do not, while the Canadian PME has also failed to show an *in vivo* androgenic response using mosquitofish in another study (Hardy, 2002). Higher concentrations of effluent and longer exposure durations for the study may

produce different results *in vivo*. Also, as already mentioned, using tissue from the same fish species (mosquitofish) for the *in vitro* assays may reduce inter-species variability and provide more consistent results. Nevertheless, caution must be taken in extrapolating *in vitro* responses to *in vivo* responses and effects.

This study also shows that AD is not likely to be the sole compound responsible for inducing masculinizing effects in wild fish as reported (Jenkins *et al.*, 2001). AD and ADD are needed to be at concentrations of about 100 μg/L to elicit gonopodium development in female mosquitofish. Given that average effluent sterol concentrations range between 100-200 μg/L, the majority of which is bound to suspended solid material in the effluent, and that microbial conversion occurs at 13% for AD and 30% for ADD under optimum conditions, it is unlikely that either of these compounds is responsible for eliciting the androgenic effects downstream from a PME discharge. Furthermore, the reported AD concentration in the river was 40 ng/L (Jenkins *et al.*, 2001), which is 2500 times lower than the minimum needed to elicit an effect in female mosquitofish.

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Appendix A: Androgen receptor binding data

sample		counts per minute		
Testosterone	0 nM	7898	7962.5	8095
	0.01 nM	8933.5	8061	8328
	0.1 nM	8125.5	8330.5	8131
	1 nM	7526	7621.1	7660.6
	10 nM	4308	4457.1	4608.6
	100 nM	1794	1829.5	1769
	1000 nM	1333.1	1238	1365
AD	0 nM	7898	7962.5	8095
	0.01 nM	8254.1	8017.1	8273.5
	0.1 nM	8214	8317.1	8404.1
	1 nM	6821	6656.6	7102.5
	10 nM	3004	2977	2754.5
	100 nM	1514.5	1481.1	1569.5
	1000 nM	1294	1275	1200.5
ADD	0 nM	7898	7962.5	8095
	0.01 nM	8514	8273.6	8702
	0.1 nM	8513.7	8524.5	7873.5
	1 nM	7678.6	6644.5	7442.6
	10 nM	3613.5	3738.6	3481.5
	100 nM	1689.5	1549.5	1372.5
	1000 nM	1181	1138.5	1189.5
estradiol	0 nM	8041.2	8447.4	7563.5
	0.01 nM	8207.1	8263.6	7868.7
	0.1 nM	7999.5	8054.5	8248.7
	1 nM	8136.5	8698.1	8151.4
	10 nM	7930.3	8357.4	8348.4
	100 nM	8225.8	8139.5	8059.0
	1000 nM	8028.5	8265.2	8086.7
T in 2% EtOH	0 nM	7898	7962.5	8095
	0.01 nM	8329.5	8563.5	8627.6
	0.1 nM	8561	8279.6	8413
	1 nM	7723.5	7472.5	7652.2
	10 nM	4553	4207	4696
	100 nM	1788	2006.5	1824.5
	1000 nM	1298.5	1189.5	1257

sample	**counts per minute**		
New Zealand PME			
Dilution factor: 0.00001	5554.5	5245.6	5387
0.0001	5416.7	5613	5237.7
0.001	5611	5479	5368.5
0.01	5502.5	5563.5	5513.5
0.1	5733.6	4750.5	4808.6
1	3977.5	5246.5	3474
New Zealand upstream reference			
Dilution factor: 0.00001	5434.5	6268	5210
0.0001	5399	5169.6	5405
0.001	5650.5	5872.5	5294
0.01	5779.5	5774.6	5366
0.1	5381	5521.6	5215.6
1	4991	3741.1	3845.7
Canadian PME			
Dilution factor: 0.00001	5237	4751	5450.5
0.0001	5554.5	5482.5	5387
0.001	5240.6	5736	5710.5
0.01	5852.6	5468.1	5032.5
0.1	4593	5340.1	4665.1
1	3166.6	3099.5	3171.5
Canadian upstream reference			
Dilution factor: 0.00001	5044.1	4928	5275.5
0.0001	5776.1	5330.5	5454.5
0.001	5265	5782	5332.1
0.01	6014	4765.2	5226.5
0.1	5363.7	5928.5	5582
1	5589.5	5124	5187.6
New Zealand STP			
Dilution factor: 0.00001	4512.1	4381.6	4265.6
0.0001	5257	5362.1	3539.7
0.001	4590	5369.5	3319.834
0.01	5128	5067.1	3398.37
0.1	5046.5	3660.1	2902.2
1	1231.5	1482	904.8

sample	**counts per minute**		
Blank			
dilution factor: 0.00001	5508	6232.5	5379.6
0.0001	5222	5596.3	5316.5
0.001	5496.1	5736.6	5440.5
0.01	5467	5916	5472.5
0.1	5582.5	5222.6	5168.5
1	5466.5	5060	4954.6

Appendix B: Aromatase inhibition activity data

sample	counts per minute		
HAD 0 ug/mL	2741.5	2821.6	2840
0.0001 ug/mL	2238	2633.7	2865.1
0.001 ug/mL	2100.6	2103	2130
0.01 ug/mL	817	801	870.5
0.1 ug/mL	334.6	365.1	325.5
1 ug/mL	193.1	225.6	238.5
10 ug/mL	379.1	219.1	191.1
New Zealand PME			
dilution factor: 0.00001	875.6	923	844.1
0.0001	852	890.1	833
0.001	963.1	886.5	884.1
0.01	927.1	906.7	951.1
0.1	888.5	928.5	935.5
1	633	844.1	663
New Zealand upstream reference			
dilution factor: 0.00001	1270.1	1225.6	1269
0.0001	1228.6	1324.5	1375.1
0.001	1318.6	1379	1352
0.01	1341.5	1333	1138.5
0.1	1170	1266.5	1389.5
1	1312.1	1421.5	1304.5
Canadian PME			
dilution factor: 0.00001	1127.5	1157.5	1164
0.0001	1173	1105.6	1123.5
0.001	1083.1	1126.5	1091
0.01	1162.6	1125.6	1157.6
0.1	1061.5	1063.5	1033.5
1	619	958.1	628.7
Canadian upstream reference			
dilution factor: 0.00001	1226.1	1243	1219.6
0.0001	1298.5	1285.5	1293
0.001	1261.7	1197.5	1285.5
0.01	1319.1	1332	1355
0.1	1310	1372.1	1232.2
1	1329.5	1228.1	1295.2

sample	**counts per minute**		
New Zealand STP			
dilution factor: 0.00001	1173.5	1165.6	1201.5
0.0001	1151	1151.5	1172.5
0.001	1170.6	1247.6	1231.1
0.01	1279.1	1206.5	1282.5
0.1	1136	1062.7	1058.7
1	586	548.7	573.5
Blank			
dilution factor: 0.00001	1940	2000	1920
0.0001	1930	2010	1963
0.001	1956	1980	1941
0.01	1920	2040	1952
0.1	1890	1992	1922
1	1855.1	1970.2	2021.5

Appendix C: Mosquitofish anal fin ray data

Methyltestosterone (500ng/L)

Tank A

			ratio 4:6
fish 1	ray 4	2291.26	1.2685
	ray 6	1806.29	
fish 2		2311.27	1.5506
		1490.54	
fish 3		2109.07	1.2792
		1648.80	
fish 4		2121.18	1.4405
		1472.50	
fish 5		1940.46	1.2421
		1562.19	
fish 6		2151.60	1.3952
		1542.19	
fish 7		2021.88	1.2732
		1588.05	
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.3499

Tank B

			ratio 4:6
fish 1	ray 4	1849.44	1.7121
	ray 6	1080.22	
fish 2		1867.60	1.3645
		1368.72	
fish 3		1858.22	1.6539
		1123.53	
fish 4		1941.19	1.2730
		1524.90	
fish 5		1854.57	1.2881
		1439.78	
fish 6	dead		
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.4583

Ethanol (negative control)

Tank A

		ratio 4:6
fish 1	ray 4 1402.16	0.9735
	ray 6 1440.34	
fish 2	1391.01	0.9372
	1484.27	
fish 3	1785.33	1.0839
	1647.10	
fish 4	1873.68	1.0930
	1714.31	
fish 5	1633.07	1.1700
	1395.78	
fish 6	1839.35	1.1499
	1599.60	
fish 7	1789.42	1.1166
	1602.56	
fish 8	1678.85	1.0515
	1596.55	
fish 9	1823.95	1.0658
	1711.36	
fish 10	dead	
	avg. ratio 4:6	1.0713

Tank B

		ratio 4:6
fish 1	ray 4 1676.29	1.0756
	ray 6 1558.44	
fish 2	1714.65	1.0855
	1579.63	
fish 3	1739.05	1.0888
	1597.23	
fish 4	1867.32	1.0765
	1734.64	
fish 5	dead	
fish 6	dead	
fish 7	dead	
fish 8	dead	
fish 9	dead	
fish 10	dead	
	avg. ratio 4:6	1.0816

AD (100 ug/L)
Tank A

			ratio 4:6
fish 1	ray 4	1422.20	1.2228
	ray 6	1163.09	
fish 2		1358.70	1.1960
		1136.07	
fish 3		1321.73	1.1161
		1184.21	
fish 4		1409.71	1.1058
		1274.79	
fish 5		1649.88	1.0526
		1567.37	
fish 6		1717.85	1.2321
		1394.21	
fish 7		1735.87	1.1712
		1482.09	
fish 8		1849.57	1.2630
		1464.42	
fish 9		1776.23	1.1808
		1504.21	
fish 10	dead		
	avg. ratio 4:6	1.1712	

Tank B

			ratio 4:6
fish 1	ray 4	1719.51	1.0855
	ray 6	1584.10	
fish 2		1696.52	1.1377
		1491.17	
fish 3		1749.19	1.0782
		1622.28	
fish 4		1769.50	1.1257
		1571.91	
fish 5		1792.77	1.2054
		1487.31	
fish 6		1777.43	1.1424
		1555.91	
fish 7		1648.68	1.1604
		1420.74	
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6	1.1336	

ADD (100 ug/L)

Tank A

			ratio 4:6
fish 1	ray 4	1785.31	1.2863
	ray 6	1387.93	
fish 2		1726.65	1.3809
		1250.38	
fish 3		1873.04	1.2487
		1499.97	
fish 4		1934.13	1.3008
		1486.87	
fish 5		1834.21	1.3814
		1327.81	
fish 6		1778.05	1.2327
		1442.43	
fish 7		1885.52	1.3222
		1426.01	
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.3076

Tank B

			ratio 4:6
fish 1	ray 4	1842.93	1.1674
	ray 6	1578.63	
fish 2		1512.27	1.1184
		1352.12	
fish 3		1850.46	1.1849
		1561.68	
fish 4		1697.03	1.2038
		1409.73	
fish 5		1824.22	1.1871
		1536.76	
fish 6		1822.58	1.1105
		1641.18	
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.1620

ADD (10 ug/L)

Tank A

			ratio 4:6
fish 1	ray 4	1390.45	1.1106
	ray 6	1251.99	
fish 2		1875.89	1.1505
		1630.53	
fish 3		1790.51	1.1140
		1607.33	
fish 4	dead		
fish 5	dead		
fish 6	dead		
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.1250

Tank B

			ratio 4:6
fish 1	ray 4	1393.32	0.9706
	ray 6	1435.57	
fish 2		1339.69	1.0012
		1338.11	
fish 3		1759.17	1.1542
		1524.16	
fish 4		1740.71	1.0713
		1624.81	
fish 5		1918.69	1.1375
		1686.74	
fish 6		1583.62	1.0594
		1494.89	
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.0657

ADD (1 ug/L)

Tank A

			ratio 4:6
fish 1	ray 4	1195.84	0.9973
	ray 6	1199.08	
fish 2		1458.46	1.1109
		1312.90	
fish 3		1256.97	0.9999
		1257.05	
fish 4		1449.97	1.0897
		1330.64	
fish 5		1708.42	1.2093
		1412.75	
fish 6			N/A
fish 7		1833.77	1.1460
		1600.20	
fish 8			N/A
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.0922

Tank B

			ratio 4:6
fish 1	ray 4	1663.87	1.1251
	ray 6	1478.84	
fish 2			N/A
fish 3		1426.38	1.1775
		1211.39	
fish 4		1579.25	1.0457
		1510.20	
fish 5		1790.18	1.2141
		1474.46	
fish 6		1778.40	1.0937
		1626.09	
fish 7		1804.24	1.1186
		1612.95	
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.1291

CA (250 ug/L)

Tank A

			ratio 4:6
fish 1	ray 4	1833.02	1.3351
	ray 6	1372.90	
fish 2		1973.88	1.3972
		1412.71	
fish 3		1901.39	1.2469
		1524.85	
fish 4		1981.13	1.3981
		1417.00	
fish 5		1844.66	1.1117
		1659.34	
fish 6		2086.99	1.1743
		1777.26	
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.2772

Tank B

			ratio 4:6
fish 1	ray 4	1997.70	1.3756
	ray 6	1452.29	
fish 2		1930.30	1.2879
		1498.84	
fish 3		1879.58	1.2172
		1544.15	
fish 4		1870.37	1.4098
		1326.70	
fish 5		1949.18	1.3604
		1432.75	
fish 6		2012.09	1.2987
		1549.29	
fish 7		1802.45	1.2589
		1431.76	
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.3155

CA (25 ug/L)

Tank A			**ratio 4:6**
fish 1	ray 4	1914.72	1.3635
	ray 6	1404.25	
fish 2		1837.30	1.4570
		1260.98	
fish 3		1935.12	1.5029
		1287.61	
fish 4		1884.06	1.3162
		1431.40	
fish 5		1892.21	1.2966
		1459.34	
fish 6		1934.89	1.3913
		1390.66	
fish 7		1966.75	1.4344
		1371.10	
fish 8		1954.99	1.4129
		1383.72	
fish 9		1777.32	1.2377
		1436.00	
fish 10		1785.07	1.1933
		1495.88	
	avg. ratio 4:6	1.3606	

Tank B

			ratio 4:6
fish 1	ray 4	1859.76	
	ray 6	1328.12	1.4003
fish 2		1990.75	
		1014.21	N/A
fish 3		1959.87	
		1316.82	1.4883
fish 4		1944.02	
		1456.83	1.3344
fish 5		1876.93	
		1343.06	1.3975
fish 6		1926.94	
		1316.23	1.4640
fish 7		1916.08	
		1396.18	1.3724
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6	1.4095	

CA (2.5 ug/L)

Tank A

			ratio 4:6
fish 1	ray 4	1841.14	1.3657
	ray 6	1348.14	
fish 2		1976.68	1.4104
		1401.54	
fish 3		1984.98	1.2079
		1643.28	
fish 4		1948.46	1.4691
		1326.25	
fish 5		1927.26	1.4269
		1350.67	
fish 6		1951.45	1.3549
		1440.30	
fish 7		2033.51	1.4402
		1412.01	
fish 8		2014.42	1.2227
		1647.47	
fish 9		1858.36	1.4911
		1246.29	
fish 10	dead		
	avg. ratio 4:6		1.3765

Tank B

			ratio 4:6
fish 1	ray 4	1901.96	1.4315
	ray 6	1328.69	
fish 2		2023.16	1.4217
		1423.03	
fish 3		2106.92	1.4727
		1430.64	
fish 4		1913.85	1.3135
		1457.08	
fish 5		1905.68	1.3915
		1369.50	
fish 6		1851.04	1.3523
		1368.84	
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.3972

HAD (100 ug/L)

Tank A

			ratio 4:6
fish 1	ray 4	1739.17	1.1316
	ray 6	1536.96	
fish 2		1727.40	1.1623
		1486.16	
fish 3		1584.11	1.1054
		1433.09	
fish 4		1774.82	1.1282
		1573.08	
fish 5		1625.21	1.1003
		1477.01	
fish 6		1763.63	1.1889
		1483.41	
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.1361

Tank B

			ratio 4:6
fish 1	ray 4	1688.36	1.0660
	ray 6	1583.76	
fish 2		1812.08	1.0907
		1661.39	
fish 3		1777.41	1.1140
		1595.50	
fish 4		1742.78	1.0692
		1630.03	
fish 5		1748.27	1.1146
		1568.57	
fish 6		1728.42	1.1240
		1537.78	
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.0964

PME

Tank A

			ratio 4:6
fish 1	ray 4	1849.37	1.1141
	ray 6	1659.96	
fish 2		1818.20	1.1472
		1584.90	
fish 3		1889.88	1.1576
		1632.52	
fish 4		1656.93	1.0975
		1509.77	
fish 5		1776.90	1.1069
		1605.30	
fish 6		1757.42	1.1040
		1591.87	
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.1212

Tank B

			ratio 4:6
fish 1	ray 4	1788.16	1.0968
	ray 6	1630.39	
fish 2		1314.89	1.0198
		1289.33	
fish 3		1835.44	1.0984
		1671.03	
fish 4		1756.52	1.1313
		1552.65	
fish 5		1653.30	1.0014
		1651.03	
fish 6		1888.36	1.1447
		1649.62	
fish 7		1830.37	1.0983
		1666.58	
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.0844

STP

Tank A

			ratio 4:6
fish 1	ray 4	1774.06	1.0910
	ray 6	1626.12	
fish 2		1593.73	1.0529
		1513.68	
fish 3		1660.50	1.1367
		1460.79	
fish 4		1695.01	1.0779
		1572.46	
fish 5		1572.18	1.1429
		1375.58	
fish 6		1666.40	1.0942
		1522.90	
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.0993

Tank B

			ratio 4:6
fish 1	ray 4	1780.28	1.1637
	ray 6	1529.88	
fish 2		1549.37	1.0307
		1503.28	
fish 3		1785.83	1.0802
		1653.27	
fish 4		1684.54	1.1275
		1494.06	
fish 5		1695.60	1.1262
		1505.63	
fish 6		1684.54	1.1416
		1475.56	
fish 7		1752.15	1.1289
		1552.10	
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.1141

Upstream ref.

Tank A

			ratio 4:6
fish 1	ray 4	1637.05	1.0368
	ray 6	1579.02	
fish 2		1747.22	1.1011
		1586.76	
fish 3		1500.80	1.0589
		1417.32	
fish 4		1777.25	1.0686
		1663.08	
fish 5		1660.19	1.0557
		1572.58	
fish 6		1805.35	1.1135
		1621.35	
fish 7		1751.85	1.0787
		1624.10	
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.0733

Tank B

			ratio 4:6
fish 1	ray 4	1634.39	1.0794
	ray 6	1514.17	
fish 2		1805.32	1.1150
		1619.16	
fish 3		1721.08	1.1027
		1560.81	
fish 4		1778.44	1.1001
		1616.68	
fish 5		1767.41	1.1193
		1579.03	
fish 6	dead		
fish 7	dead		
fish 8	dead		
fish 9	dead		
fish 10	dead		
	avg. ratio 4:6		1.1033

Appendix D: *In vitro* testosterone levels RIA data

Testosterone RIA standard curve data:

conc. (pg/tube)	counts per minute		
0	1319.1	1321.5	1383.0
1.56	1105.5	1056.2	1102.5
3.125	1001.6	1038.0	1006.5
6.25	1129.6	1053.1	934.0
12.5	838.5	934.0	767.0
25	856.0	565.5	624.5
50	398.5	411.1	430.0
100	243.0	264.0	290.5
200	184.5	181.5	191.5
400	151.1	139.2	143.5
800	129.5	133.5	121.5
80000	78.6	64.1	94.5

sample	counts per minute		total weight of fish (mg)
HAD1A	301.0	295.5	348
HAD2A	312.6	332.5	532
HAD3A	492.5	540.5	636
HAD4A	343.5	336.0	589
HAD5A	656.6	579.6	731
HAD1B	325.5	291.5	990
HAD2B	190.5	185.0	348
HAD3B	256.7	261.6	603
HAD4B	272.0	261.0	660
HAD5B	759.0	509.1	731
HAD6B	546.1	476.1	600
upstream1A	615.5	596.1	434
upstream2A	331.0	352.5	243
upstream3A	338.1	292.1	548
upstream4A	602.7	625.0	679
upstream5A	638.1	594.5	829
upstream6A	862.5	830.0	899
upstream1B	359.5	394.5	523
upstream2B	878.0	921.6	696
upstream3B	368.1	354.0	551
upstream4B	1194.5	1164.5	1181

sample	counts per minute		total weight of fish (mg)
ETOH1	284.1	303.0	186
ETOH2	371.5	358.5	419
ETOH3	875.5	786.6	851
ETOH4	1357.2	1294.0	1263
PME1A	864.5	863.6	1060
PME2A	790.0	776.0	530
PME3A	566.0	551.0	554
PME4A	1516.5	1535.0	421
PME5A	579.0	608.1	530
PME6A	1509.6	1547.5	1075
PME1B	1552.6	1455.0	383
PME2B	665.0	702.5	376
PME3B	967.0	923.7	440
PME4B	1265.1	1304.5	542
PME5B	598.0	577.6	670
PME6B	1218.2	1297.6	822
PME7B	918.5	892.5	1008
STP1A	1335.5	1306.6	459
STP2A	1184.0	1173.5	562
STP3A	1112.1	1113.5	593
STP4A	1452.0	1166.0	762
STP5A	1328.5	1362.5	921
STP6A	1337.5	1384.0	1153
STP1B	1131.0	1056.7	450
STP2B	1226.1	1142.5	462
STP3B	826.1	913.6	606
STP4B	1438.0	1425.0	608
STP5B	1502.0	1396.5	702
STP6B	1280.1	1320.0	815
STP7B	1280.1	1320.0	1158

sample code: treatment/fish number/tank A or B

Appendix E: *In vitro* 17-β-estradiol levels RIA data

Estradiol RIA standard curve data:

Conc. (pg/tube)	**counts per minute**		
0	1653.0	1597.5	1709.0
1.56	1424.6	1541.5	1568.1
3.125	1424.0	1453.5	1494.0
6.25	1420.0	1444.6	1378.6
12.5	1157.6	1174.5	1064.1
25	664.5	810.5	857.0
50	611.6	597.5	470.5
100	425.5	405.0	419.7
200	290.5	305.6	306.0
400	242.5	234.0	231.1
800	223.1	243.5	215.6
80000	223.5	248.0	224.0

sample	**counts per minute**		**total weight of fish (mg)**
HAD1A	643.5	665.6	348
HAD2A	708.6	742.0	532
HAD3A	1075.5	1147.0	636
HAD4A	1094.1	1004.5	589
HAD5A	1247.0	1162.6	731
HAD1B	808.1	823.6	990
HAD2B	558.0	588.0	348
HAD3B	598.1	602.0	603
HAD4B	711.0	720.1	660
HAD5B	1384.0	1408.1	731
HAD6B	1234.5	1230.1	600
upstream1A	1021.1	1356.0	434
upstream2A	497.0	476.5	243
upstream3A	686.5	661.0	548
upstream4A	1218.6	1422.6	679
upstream5A	1188.0	1283.6	829
upstream6A	1503.5	1266.7	899
upstream1B	1003.1	912.1	523
upstream2B	1417.6	1413.0	696
upstream3B	752.0	677.7	551
upstream4B	1663.0	1639.5	1181

sample	counts per minute		total weight of fish (mg)
ETOH1	465.5	470.5	186
ETOH2	611.0	551.0	419
ETOH3	1240.5	1385.1	851
ETOH4	1853.0	1871.6	1263
PME1A	1037.5	1004.0	1060
PME2A	611.5	596.5	530
PME3A	701.0	674.5	554
PME4A	1862.0	1757.0	421
PME5A	705.0	705.5	530
PME6A	1881.0	1849.6	1075
PME1B	1863.5	1847.0	383
PME2B	662.0	770.6	376
PME3B	768.0	806.0	440
PME4B	1754.2	1685.5	542
PME5B	753.5	749.7	670
PME6B	1263.7	1314.5	822
PME7B	1038.5	1054.0	1008
STP1A	1702.1	1672.0	459
STP2A	1378.0	1283.5	562
STP3A	768.2	747.6	593
STP4A	1148.0	1214.5	762
STP5A	1494.0	1472.5	921
STP6A	1367.5	1294.6	1153
STP1B	904.8	903.5	450
STP2B	652.1	713.5	462
STP3B	825.6	846.5	606
STP4B	1183.5	1122.0	608
STP5B	1144.0	1138.5	702
STP6B	1525.1	1516.0	815
STP7B	1488.1	1581.0	1158

sample code: treatment/fish number/tank A or B

Appendix F: List of abbreviations

AD	androstenedione
ADD	1,4-androsta-diene-3,17-dione
AR	androgen receptor
CA	cyproterone acetate
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GTH	gonadotropin hormone
HAD	4α-hydroxyandrostenedione
HPG	hypothalamus-pituitary-gonads
HRE	hormone response element
IPCS	International Programme on Chemical Safety
LH	luteinizing hormone
MT	methyl-testosterone
PME	pulp and paper mill effluent
RBA	relative binding affinity
STP	sewage treatment-plant effluent
VTG	vitellogenin