# ESTROGENIC AND ANDROGENIC POTENTIAL OF MUNICIPAL SEWAGE IN AUSTRALIA AND NEW ZEALAND

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A thesis
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by

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Abstract of a thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

**Estrogenic and androgenic potential of municipal sewage in Australia and New Zealand**

**by F.D.L Leusch**

Studies in Europe, Japan, and North America have reported that wild fish exposed to treated sewage effluents can exhibit significant physiological and reproductive abnormalities consistent with exposure to hormonally active chemicals. The main objective of this research project was to examine the estrogenic and androgenic activity in treated sewage to determine the risk associated with treated sewage discharges in Australia and New Zealand.

Several bioassays, including a sheep estrogen receptor and a rainbow trout androgen receptor binding assay, were set up and validated with model compounds. The assays were then used to measure the estrogenic and androgenic activity in sewage samples from 15 municipal sewage treatment plants (STP) utilizing a variety of treatment technologies. Raw sewage samples contained high levels of both estrogenic and androgenic activity, up to 185 ng/L estradiol equivalents (EEq) and up to 9330 ng/L testosterone equivalents (TEq), respectively. Secondary treatment processes such as activated sludge had the greatest impact on removal of biological activity from the wastewater. The estrogenic and androgenic activity in final treated effluents were <1 to 4.2 ng/L EEq and <6.5 to 736 ng/L TEq, respectively. Based on lowest observable effective concentrations reported in the literature, these levels are unlikely to induce biological effects in exposed fish in the short term.

To examine potential long-term effects, resident mosquitofish chronically exposed to undiluted treated sewage were sampled. Several morphological biomarkers indicative of endocrine disruption were measured and compared with mosquitofish captured at a reference site. Mosquitofish captured in a constructed wetland for tertiary treatment of secondary-treated sewage exhibited morphological differences such as elongated anal fins consistent

with exposure to androgenic chemicals, although this effect was not measurable in fish collected at sites further downstream or at any of the other sites. Based on these results, it is unlikely that mosquitofish populations would be significantly affected by exposure to final treated sewage. A reverse transcription real-time polymerase chain reaction (RT-PCR) method to measure the production of a female-specific protein (vitellogenin) mRNA in adult male mosquitofish was developed, and this could be used as a rapid test to detect early changes in individuals exposed to estrogenic activity.

**Keywords:** activated sludge, androgen receptor (AR), biomarkers, estrogen receptor (ER), *in vitro* bioassay, mosquitofish, rainbow trout, receptor binding assays, sewage treatment, sheep, trickling filter, vitellogenin

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# Table of Contents

# List of Figures

# List of Tables

# List of Abbreviations

1°Treat = Primary-treated sewage

2°Treat = Secondary-treated sewage

4:6 ratio = Ratio of the length of the $4^{th}$ anal fin to the $6^{th}$ anal fin

AGAL = Australian Government Analytical Laboratory

ANCOVA = Analysis of covariance

ANOVA = Analysis of variance

AR = Androgen receptor

AS = Activated sludge bioreactor

bp = Base pairs

BDL = Below detection limit

$B_{max}$ = Total number of binding sites

BNR = Biological and nutrient removal

CAS RN = Chemical abstracts registry number

CC = Chlorine contact tanks

Ct = Threshold cycle

$\Delta$Ct = Difference in Ct between target gene and housekeeping gene

$\Delta\Delta$Ct = Difference in $\Delta$Ct between control and treatment

DHT = Dihydrotestosterone

DMEM = Dulbecco's modified Eagle's medium

DNA = Deoxyribonucleic acid

DND = Did not displace

$E_1$ = Estrone

$E_2$ = 17$\beta$-Estradiol

$E_3$ = Estriol

$EC_{50}$ = Effective concentration for 50% displacement

EDC = Endocrine disrupting compound

$EE_2$ = Ethinylestradiol

EEq = Estradiol equivalents

ELISA = Enzyme linked immunosorbent assay

EP = Equivalent people

ER = Estrogen receptor

$EV_{50}$ = Equivalent volume for 50% displacement

FAM = Carboxyfluorescein

Fin Eff = Final effluent

GCMS = Gas chromatography / mass spectrometry

GGC = Grit and grease chamber

GP4 = Length of the $4^{th}$ anal fin

GPx = Length difference between the $4^{th}$ and $6^{th}$ anal fin

GSI = Gonadosomatic index

HEPES = N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid

HP = Holding pond

JOE = Carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein

$K_d$ = Dissociation constant

LOEC = Lowest observable effective concentration

MDL = Method detection limit

MS222 = Tricaine methanesulfonate

NA = Data not available

NEAA = Non essential amino acids

NP = Nonylphenol

NT = Not tested

OP = Octylphenol

PCR = Polymerase chain reaction

PE = Proliferative effect

Plt Eff = Plant effluent

POPOP = 1,4-bis[2-5-phenyloxazolyl]benzene

PPO = 2,5,-diphenyloxazole

PS = Primary settling tank

RBA = Relative binding affinity

Ref = Reference site

RNA = Ribonucleic acid

RPE = Relative proliferative effect

RPP = Relative proliferative potency

RT = Reverse transcription

RT-PCR = Reverse transcription real-time polymerase chain reaction

S = Screens

SD = Standard deviation

SE = Standard error

SPE = Solid-phase extraction

SRB = Sulforhodamine B

SS = Secondary settling tank

STP = Sewage treatment plant

STP A (in Chapter 6) = Sewage treatment plant F1A in Chapter 5

STP B (in Chapter 6) = Sewage treatment plant S11A in Chapter 5

T = Testosterone

TEq = Testosterone equivalents

TIE = Toxicity identification evaluation

TF = Trickling filter

Tm = Melting temperature

UK = United Kingdom

USA = United States of America

UV = Ultraviolet

VBA = Visual basic

Vtg = Vitellogenin

W = Wetland

Wt = Wetland

# 1. Introduction

## 1.1. Endocrine disrupting compounds (EDCs)

The endocrine system is a biochemical communication system that regulates body function and responses via chemical messengers, the hormones. Exposure to even low levels of hormonally active agents (termed endocrine disrupting compounds, or EDCs) can result in significant dysfunctions of the endocrine system in a wide range of species from different animal phyla (Crisp et al. 1998, Depledge and Billinghurst 1999, IEH 1999, Taylor and Harrison 1999, WHO 2002, Miyamoto and Burger 2003).

## 1.2. EDCs in sewage water

Fish exposed to domestic sewage or sewage-polluted rivers exhibit reproductive dysfunctions, often associated with estrogenic stimulation, such as production of vitellogenin (Vtg) in males. Vtg is a precursor protein to egg-yolk, and its production is mainly modulated in the liver under estrogenic stimulation. These findings have been confirmed by research teams in the UK (Purdom et al. 1994, Lye et al. 1997, Jobling et al. 1998, Rodgers-Gray et al. 2001), continental Europe (Viganò et al. 2001, Solé et al. 2002), and North America (Folmar et al. 1996, McArdle et al. 2000). Three hormones in sewage treatment plant (STP) effluents have been identified as responsible for the majority of the estrogenic effect, namely 17β-estradiol ($E_2$), oestrone and the synthetic hormone ethynylestradiol ($EE_2$) (Desbrow et al. 1998, Routledge et al. 1998, Belfroid et al. 1999). Nonylphenol, a degradation product of alkylphenolic compounds with estrogenic activity, has also been detected in industrial wastewater (Desbrow et al. 1998, Sheahan et al. 2002b).

Identification of the point source of estrogenic and/or androgenic effects and of the chemicals that cause such effects is a critically important research venture. This would allow implementation of better treatment technologies and/or tighter discharge limits for these chemicals, which can significantly improve the quality of the STP effluents (Sheahan et al. 2002a).

While most of the studies on endocrine disruption in sewage focus on compounds with estrogenic activities, androgenic hormones have also been found in river systems receiving STP effluents (Thomas et al. 2002), and could contribute to endocrine disruption.

## 1.3. Aims and objectives

The main purpose of this research was to determine estrogenic and androgenic activity in sewage in Australia and New Zealand and to examine exposed fish populations for signs of endocrine disruption. This was addressed using an effects-based approach with bioassays based on different levels of biological organization in five objectives, listed below.

- **To develop bioassays to measure endocrine disruption**

The first objective was to develop a battery of bioassays to measure estrogenic and androgenic activity of water samples using local resources. Appropriate methods to extract organics from wastewater samples were also developed under this objective (Chapter 3).

- **To determine estrogenic and androgenic activity in treated sewage**

Once the bioassays were developed, they were used to determine the level of estrogenic and androgenic activity associated with treated sewage (Chapter 5)

- **To compare the efficacy of treatment processes**

We also wanted to compare the efficacy of different sewage water treatment technologies currently in use in Australia and New Zealand at removing estrogenic activity during treatment to identify steps of the treatment train that were most effective (Chapter 4 and 5).

- **To evaluate potential environmental impacts of EDCs on fish**

If chemicals with estrogenic and androgenic activity were present in treated sewage water, it was necessary to examine biomarkers of exposure to estrogenic or androgenic chemicals to determine if exposed fish populations were significantly affected by exposure to treated sewage (Chapter 6).

- **To develop a more sensitive biomarker of exposure in mosquitofish**

We were also determined to develop a new method to measure Vtg mRNA in adult male mosquitofish to provide a rapid and sensitive biomarker of exposure to estrogenic chemicals, suitable for caging studies. The assay would not be used in this study, but would be available for future work in the area (Chapter 7).

## 1.4. Hypotheses

- **EDCs are present in sewage in Australia and New Zealand**

Because EDC release in the environment appear to correlate with industrial, agricultural, and residential development, it was hypothesized that EDCs would be present in treated sewage water and rivers in Australia and New Zealand, in concentrations similar to those found in countries of comparable socio-economic development (Chapter 4 and 5). Based on the literature, hormones and alkylphenolic degradation products were expected to be the most biologically-active EDCs in sewage water (Desbrow et al. 1998, Snyder et al. 2001, Thomas et al. 2002).

- **Activated sludge treatment removes most activity of EDCs from sewage**

Based on physico-chemical characteristics of suspected EDCs, it was hypothesized that activated sludge treatment would be the most effective step in removing these chemicals from the wastewater, due to their high sorption potential to sludge (Chapter 4 and 5).

- **Fish exposed to treated sewage exhibit signs of exposure to EDCs**

A recent study in New South Wales, Australia, showed that fish caught directly downstream of a STP point discharge exhibited morphological abnormalities consistent with exposure to reproductive endocrine disruptors (Batty and Lim 1999). It was therefore hypothesized that fish directly exposed to STP effluents would exhibit signs of endocrine disruption (Chapter 6).

- **Exposure to estrogens rapidly induces Vtg mRNA within days**

Based on studies with other fish species (Islinger et al. 2002), it was hypothesized that significant Vtg mRNA induction in adult male mosquitofish would occur within 7 days of exposure to environmentally-relevant concentrations of estrogens (Chapter 7).

## 1.5. Thesis format

The chapters in this thesis are organized as stand-alone scientific papers. This has led to some overlap in the material and methods section (particularly between Chapters 3, 4, and 5).

Some material has been intentionally left out of the general introduction and literature review to avoid repetition in the introductions to data chapters. The specific discussions in each data chapter (Chapters 3 to 7) contain most of the discussion material, while a more concise general discussion at the end is aimed to raise synergies between the different chapters and to show the coherence of different chapters to the overall purpose of the research. It also addresses some of the limitations of this study. It too has been intentionally kept short to avoid repetitions.

# 2. Literature review

## 2.1. Endocrine system

The endocrine system is composed of diverse glands which control hormone metabolism. Hormones in turn regulate a variety of biological functions including growth, metabolism, tissue function and differentiation, sexual development and behaviour, and development of the immune system (Hadley 1988). Most hormones bind to specific membrane receptors on target cells, triggering a cascade of biochemical reactions that eventually lead to the intended effect (*e.g.* synthesis of a specific protein, or development of a certain tissue type). Some lipophilic hormones (such as steroid and thyroid hormones) however bind directly to intracellular receptors. This receptor-hormone complex interacts with transcription-control sequences of the DNA, thus modulating RNA and protein synthesis of specific genes (Fig. 2.1) (Hadley 1988, Lodish et al. 1995, Zacharewski 1997).

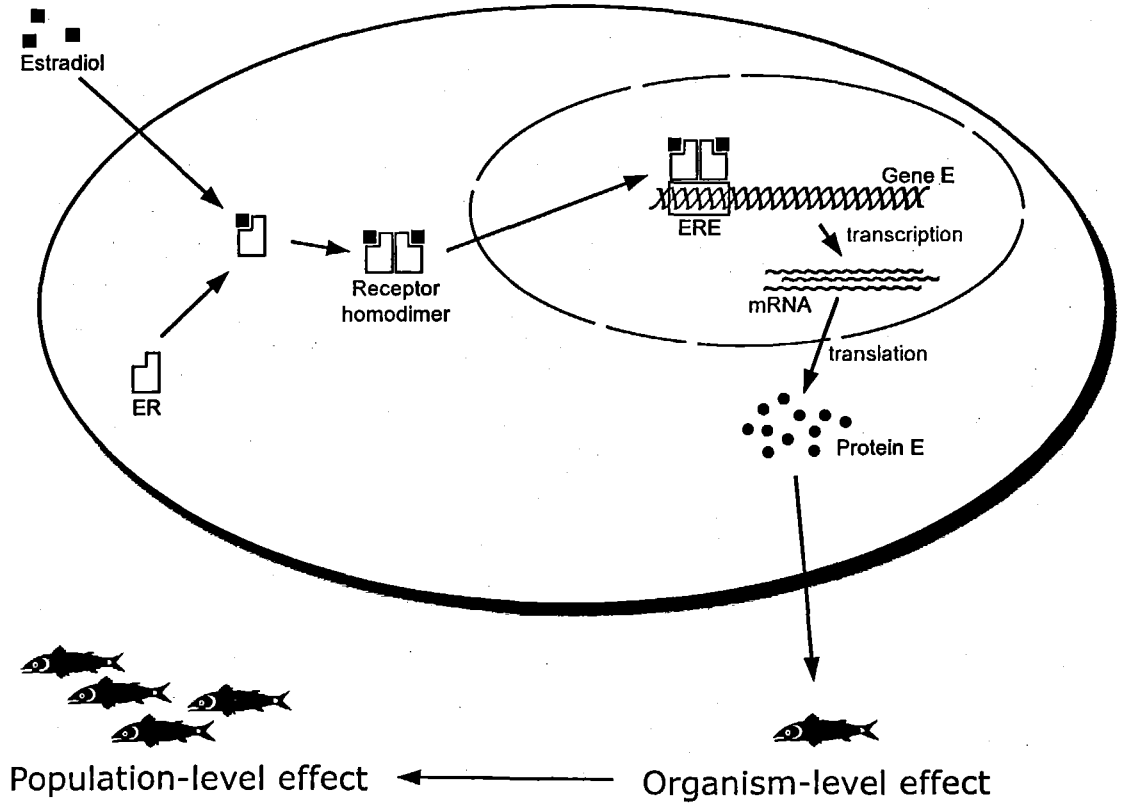


**Figure 2.1:** Mechanism of action of lipophilic hormones (eg steroids) with intracellular receptor targets.

## 2.2. Endocrine disruption

A variety of synthetic chemicals ranging from insecticides to industrial chemicals have been reported to have endocrine-disrupting effects (Colborn et al. 1993, Cheek et al. 1998, Johnson and Jürgens 2003). However, only a fraction of all synthetic chemicals released in the environment have been tested for endocrine disruption (Jobling et al. 1995, NIEHS 2002). Organisms in aquatic environments are especially susceptible to pollutants, since contaminants can accumulate via both waterborne (through the gills) and food web routes of exposure. Fish reproduction is sensitive to environmental factors, and is particularly vulnerable to EDCs (Sumpter 1997, Kime 1999, Jalabert et al. 2000). Fish exposed to industrial effluents exhibit a wide array of abnormalities of the reproductive or thyroidal systems (Van Der Kraak et al. 1992, Hontela et al. 1995, Munkittrick et al. 1998, Zhou et al. 2000, Jobling and Tyler 2003).

## 2.3. Mechanisms of endocrine disruption

The endocrine system is regulated by a complicated array of feedback mechanisms, and EDCs can affect homeostasis by interfering at several levels of the endocrine axis (WHO 2002). At the molecular level, EDCs can bind to hormone-specific receptors, thus hindering the normal hormonal response. But EDCs can also affect the endocrine system in other ways, for example by affecting the synthesis of the hormone receptors themselves or interferring with the metabolism of the natural hormone (WHO 2002). The hormone mimics present in sewage generally affect reproduction mostly through binding to hormone receptors, and the bioassays developed in this thesis therefore focus on receptor-mediated effects.

## 2.4. Endocrine disruption in sewage

Fish exposed to domestic sewage or sewage-polluted rivers exhibit a wide array of reproductive abnormalities, including abnormally high levels of Vtg in males, high incidences of intersexuality in gonochoristic species, and altered endocrine status (Christiansen et al. 2002, Jobling and Tyler 2003).

### 2.4.1. *Vitellogenin induction in male fish*

In a study at 15 sewage treatment plants (STP) in the United Kingdom (UK), male rainbow trout (*Oncorhynchus mykiss*) caged for 3 weeks in STP effluents had significantly higher (up to 100,000-fold) plasma Vtg compared with control fish (Purdom et al. 1994). Vitellogenin induction was also observed in male carp (*Cyprinus carpio*), although to a lesser extent than in trout. Vitellogenin is a glycolipophosphoprotein precursor to egg-yolk synthesized by the liver of oviparous vertebrates under estrogenic stimulation (Denslow et al. 1999). It is normally only produced in significant quantities in females, and only detected at very low levels in males. Under normal circumstances, circulating estrogen concentrations in males are too low to trigger significant expression of the Vtg gene (Sumpter and Jobling 1995), but exposure to xenoestrogens can greatly induce Vtg production in males (Hansen et al. 1998).

Elevated plasma Vtg levels in male rainbow trout and carp caged in STP effluents in the UK (Purdom et al. 1994, Sumpter and Jobling 1995, Harries et al. 1996) suggested the presence of chemicals with estrogenic activity in treated sewage. Laboratory studies have also reported significant Vtg induction in larval (Todorov et al. 2002) and juvenile (McArdle et al. 2000) sunshine bass (*Morone saxatilis*), adult male fathead minnows (*Pimephales promelas*) (Hemming et al. 2004), and male rainbow trout (Knudsen et al. 1997, Harries et al. 1999, Nakari 2004) exposed to STP effluents, confirming the estrogenicity of treated sewage. Field sampling indicated that male fish captured near STP had high levels of Vtg. Adult male carp and walleye (*Stizostedion vitreum*) collected in the effluent channel of a municipal STP in Minnesota (USA) had high levels of plasma Vtg compared with other sites (Folmar et al. 1996, Folmar et al. 2001a); male longear sunfish (*Lepomis megalotis*) captured downstream of a municipal STP in Oklahoma (USA) had significantly elevated plasma Vtg compared with a reference site (Porter and Janz 2003); male feral carp (*C. carpio*) downstream of a Spanish STP had increased plasma Vtg compared with other sites (Petrovic et al. 2002); and wild roach (*Rutilus rutilus*) captured downstream of STP effluent discharges in UK rivers had significantly higher plasma Vtg than fish upstream of the STP outfalls (Jobling et al. 1998). Vitellogenin induction has also been reported in several marine species (Matthiessen 2003). Plasma Vtg levels in male flounder (*Platichthys flesus*) collected in the Tyne river estuary (which receives effluent from a major STP) were 15 - 20× higher than in males from a reference site (Lye et al. 1997); male flounder (*Pleuronectes yokohamae*) captured from Tokyo Bay (which receives large amounts of industrial and domestic sewage effluent) had significantly higher plasma Vtg than fish from a reference site (Hashimoto et al. 2000); and

male bigmouth sole (*Hippoglossina stomata*), English sole (*Pleuronectes vetulus*), and hornyhead turbot (*Pleuronichthys verticalis*) sampled near an STP effluent outfall had high levels of Vtg, with a trend toward higher values close to the outfall (Roy et al. 2003).

Vitellogenin, as precursor to egg-yolk, has no known target cell or function in males, and remains in the plasma until it is degraded by plasma proteases (Denslow et al. 1999). High levels of plasma Vtg have been linked to liver failure (Folmar et al. 2001b), but it is unclear if high levels of Vtg in males impact population fitness.

### *2.4.2. Intersexuality in fish*

Intersex is characterized by the presence of both male and female sex cells in the gonads of an individual (ovotestis), and/or the presence of an ovarian cavity in male testes. A survey of roach (*R. rutilus*) in 8 rivers in the UK reported a high incidence of intersexuality in wild populations, particularly in populations captured downstream of sewage treatment plant (STP) outfalls (Jobling et al. 1998). This abnormal occurrence of hermaphroditism was apparent in 16 - 100% of all test sites, compared with 4 - 18% in the reference populations (Jobling et al. 1998). The most severely impacted populations (where 100% of all examined phenotypic males showed some level of intersexuality) were from sites immediately downstream of STP discharges. The authors also reported a significant relationship between the proportion of intersex fish and the concentration of the STP effluent in the river, suggesting that compounds in treated sewage were responsible for inducing intersex in this species of fish. There was a good correlation between plasma Vtg and the severity of intersex, suggesting that plasma Vtg may be a good indicator of the level of gonadal disruption in that species (Jobling et al. 2002). Intersex has also been reported in other species in rivers with a high input of treated sewage, including gudgeon (*Gobio gobio*) (van Aerle et al. 2001), barbel (*Barbus plebejus*) (Viganò et al. 2001), and bream (*Abramis brama*) (Vethaak et al. 2002). Male mosquitofish (*Gambusia holbrooki*) collected in sewage-contaminated water in New South Wales (Australia) exhibited feminization of secondary sexual characteristics consistent with exposure to estrogenic chemicals (Batty and Lim 1999). Roach exposed to STP effluent exhibited a dose-dependent and persistent disruption of gonadal duct development (Rodgers-Gray et al. 2001), clearly demonstrating that exposure to treated sewage could induce intersex in this species. Intersex "male" roach from an STP effluent-impacted river had reduced milt volume and sperm density compared with reference males, and severely affected fish had occluded reproductive

ducts which prevented the release of gametes, suggesting that reproductive success of intersex fish is likely to be compromised (Jobling et al. 2002).

### 2.4.3. *Other effects in fish related to altered endocrine status*

Fish exposed to STP effluents exhibit changes in plasma steroid concentrations and reproductive status, but the effects are less consistent and may vary with effluent chemistry and among species. For example, male sunfish (*L. megalotis*) collected downstream of an STP had elevated plasma testosterone (T) concentrations compared with males from a reference site (Porter and Janz 2003); female roach (*R. rutilus*) sampled in two UK rivers that receive STP discharges had significantly depressed plasma $E_2$ levels compared with reference females (Jobling et al. 2002); male carp (*C. carpio*) and walleye (*S. vitreum*) captured in the effluent channel of an STP in Minnesota (USA) had depressed plasma T compared to males at other sites (Folmar et al. 1996, Folmar et al. 2001a), and female walleye had greatly elevated plasma $E_2$ concentrations (Folmar et al. 2001a); and rainbow trout (*O. mykiss*) exposed to treated sewage had depressed plasma 11-ketotestosterone levels in males and increased $E_2$ levels in females, as well as increased gonadal weight (Höger et al. In press).

Although most effects observed in fish exposed to STP effluents appear to be consistent with exposure to estrogenic chemicals, some masculinization of mosquitofish (*Gambusia affinis*) captured downstream of an STP in Alabama (USA) has also been reported (Angus et al. 2002). In that study, male mosquitofish downstream of the STP discharge had slightly elongated anal fins (an indicator of exposure to androgenic chemicals in that poeciliid species) and significantly larger testis than fish from a reference site.

### 2.4.4. *Effects in other aquatic species*

There is evidence that invertebrates exposed to STP effluents exhibit morphological abnormalities consistent with exposure to estrogenic chemicals as well. Mussels (*Elliptio complanata*) had significantly higher hemolymph and gonadal Vtg after exposure to STP effluents in the laboratory, and mussels caged downstream of an STP discharge in Quebec (Canada) had significantly higher Vtg than those at the upstream site (Gagné et al. 2001); a significant number of *Gammarus pulex* females captured downstream of an STP in the UK displayed an abnormal structure of oocytes in vitellogenesis (Gross et al. 2001); and

intersexuality in harpacticoid copepods was correlated with exposure to STP effluents (Moore and Stevenson 1991, 1994).

## 2.5. Evaluation of EDCs in sewage water

Endocrine disrupting activity of sewage water can be evaluated in a variety of ways.

### 2.5.1. *In vitro* bioassays

Several *in vitro* bioassays have been developed to measure the estrogenic and androgenic activity of simple compounds or complex mixtures. Chemicals can interfere with endocrine systems in several ways, including binding to the hormone receptors, affecting the synthesis or metabolism of natural hormones, and affecting the synthesis or metabolism of the hormone receptor itself (Zacharewski 1997, Katzenellenbogen and Muthyala 2003). There are three main categories of assays, depending on which endpoint of the biological response to natural steroids they measure (Kinnberg 2003). Each assay has its advantages and limitations, and no single assay can provide a complete assessment of the endocrine-disrupting activity of a chemical or mixture.

Competitive receptor binding assays measure the ability of chemicals to compete with the native hormone for binding to the receptor (ICCVAM 2003). Binding to the receptor is the initial step of genomic steroid action (Danzo 1997) and is a prerequisite for many subsequent cellular effects (Fig. 2.1), such as Vtg synthesis (Flouriot et al. 1997). Several receptor binding assays have been developed to assess estrogenic and androgenic activity. Estrogen receptors (ER) have been isolated from rat (*Rattus norvegicus*) uteri (ICCVAM 2003), alligator (*Alligator mississipiensis*) oviducts (Vonier et al. 1996), and livers of Atlantic salmon (*Salmo salar*) (Lazier et al. 1985, Yadetie et al. 1999), carp (*C. carpio*) (Kloas et al. 2000), and frog (*Xenopus laevis*) (Lutz and Kloas 1999). Androgen receptors (AR) have been isolated from Atlantic croaker (*Micropogonias undulatus*) brain and ovaries (Sperry and Thomas 1999a), kelp bass (*Paralabrax clathratus*) brain and ovaries (Sperry and Thomas 1999b), rainbow trout (*O. mykiss*) brains (Wells and Van Der Kraak 2000), and goldfish (*Carassius auratus*) brain, ovaries, and testes (Wells and Van Der Kraak 2000). A large number of natural and synthetic chemicals with a range of chemical structures can bind to

these receptors (Katzenellenbogen 1995, Hong et al. 2002, Katzenellenbogen and Muthyala 2003). Other receptors such as progesterone, thyroid, and retinoid receptors have also been isolated from animal tissues, and exogenous compounds have the ability to displace native ligands from these receptor sites (Katzenellenbogen and Muthyala 2003). Although binding to the receptor clearly identifies a chemical as a potential hormone mimic, it does not necessarily imply effects at the organism-level (ICCVAM 2003, Yamasaki et al. 2004). Receptor binding assays can therefore be poor predictors of more complex *in vitro* and *in vivo* responses (Kinnberg 2003). Receptor binding assays also cannot distinguish between receptor agonists and antagonists (Zacharewski 1998).

Receptor-mediated gene induction can be measured in gene expression and reporter gene assays. Reporter gene assays are conducted with genetically engineered yeast (Routledge and Sumpter 1996, Sohoni and Sumpter 1998, Garcia-Reyero et al. 2001), fish (Ackermann et al. 2002, Rutishauser et al. 2004), or mammalian cells (Legler et al. 1999, Vinggaard et al. 1999, Balaguer et al. 2000) transfected with receptor DNA binding domain linked to a reporter gene (Zacharewski 1997, Kinnberg 2003). Binding of an agonist to the receptor causes a conformational change that allows it to bind to the receptor DNA binding domain and activate the gene expression machinery (Fig. 2.1). The product of transcription of the reporter gene can then be measured appropriately (most reporter genes are galactosidase or luciferase genes, with protein products easily measured by spectrophotometry and luminometry). Estrogen and androgen reporter gene assays are susceptible to the presence of anti-estrogenic or anti-androgenic substances, respectively, which in complex mixtures can counteract the effects of agonistic chemicals and result in underestimation of the activity of the sample (Kinnberg 2003). Also, chemicals can have non-genomic receptor-mediated effects (Thomas 2003), which would be missed in reporter gene assays. Gene expression assays measure gene induction in unmodified cells, such as Vtg induction in rainbow trout (*O. mykiss*) hepatocytes (Petit et al. 1997, Tremblay and Van Der Kraak 1998).

The E-Screen assay measures the estrogenic potency of a chemical or mixture to induce cell proliferation in breast cancer cells (MCF-7 cells; Soto et al. 1995, Körner et al. 1999). Although this assay provides a measure of estrogenic activity at the cellular levels incorporating both genomic and nongenomic receptor-mediated effects, there is considerable variation between different cell lines (Villalobos et al. 1995). The E-Screen assay is also more expensive and time-consuming than other assays, limiting its application to large-scale screening programs (Kinnberg 2003).

Ideally, a battery of complimentary bioassays should be used to assess estrogenic and/or androgenic activity of environmental samples. A combination of bioassays can provide insights into the mechanisms of action of specific endocrine disruptors (Zacharewski 1997). However, *in vitro* bioassays can only provide limited information on the potential of a specific chemical or mixture to induce whole-organism effects, and it is therefore important to correlate *in vitro* results with *in vivo* measurements (Zacharewski 1998). This is particularly true when dealing with endocrine disruptors that can act in unpredictable ways in whole organism systems due to the complexity of feedback mechanisms involved in endocrine communication (Hadley 1988). Recent reviews by environmental policy agencies (Huet 2000, NIEHS 2002, ICCVAM 2003) suggest a range of *in vitro* and *in vivo* bioassays to determine the endocrine-disrupting potential of simple chemicals or complex environmental samples.

### 2.5.2. *In vivo* bioassays

Several endpoints can be used as indicators of endocrine disruption in fish. The most widely used biomarker of exposure to estrogenic chemicals in fish is Vtg induction (Denslow et al. 1999). Levels of Vtg can be measured using enzyme-linked immunosorbent assay (ELISA) in many different species of fish (Korsgaard and Pedersen 1998, Lomax et al. 1998, Sherry et al. 1999, Folmar et al. 2000, Folmar et al. 2001a, Holbech et al. 2001, Pait and Nelson 2003, Ataria et al. 2004). Vtg induction can also be quantified by measuring the levels of Vtg mRNA (Bowman and Denslow 1999, Denslow et al. 2001, Lattier et al. 2001, Islinger et al. 2002). Induction of specific genes can also be used to determine if animals have been exposed to estrogenic and/or androgenic chemicals, and can be measured by novel techniques such as microarrays (Larkin et al. 2002) or differential display reverse transcription polymerase chain reaction (Denslow et al. 2001). Circulating levels of plasma steroids and relative gonad size (GSI) have also been measured in fish exposed to sewage effluent (Folmar et al. 2001a, Angus et al. 2002, Jobling et al. 2002) and provide a direct assessment of their endocrine status. Morphological endpoints can also be used to determine exposure to endocrine disruptors. Ovotestis (the presence of both oocytes and testicular tissue in gonads of the same individual) and intersex have been used as an indicator of exposure to EDCs (Bortone and Davis 1994, Jobling et al. 1998), with a well described histopathology (Nolan et al. 2001). Development of secondary sexual characteristics in fish is mostly directed by

androgens and estrogens (Hadley 1988, Bond 1996), and abnormal development of secondary sexual characteristics in mosquitofish (*Gambusia affinis and G. holbrooki*) has been used as an indicator of exposure to chemicals with androgenic and estrogenic activity (Denton et al. 1985, Drèze et al. 2000, Angus et al. 2001, Doyle and Lim 2002).

### 2.5.3. Toxicity identification evaluation (TIE) of EDCs in sewage

Exposure to sewage induces several endocrine-related changes in fish (reviewed in section 2.1). *In vitro* bioassays revealed high levels of estrogenic activity in treated sewage. Levels of 0.2 to 25 ng/L of estradiol equivalents (EEq) in several studies in Germany with the E-Screen (Körner et al. 1999, Körner et al. 2000, Körner et al. 2001), <0.1 to 62 ng/L EEq in the Netherlands with a combination of reporter gene assays and receptor binding assays (Murk et al. 2002), <3 to 13 ng/L in the United Kingdom with yeast reporter gene assay (Kirk et al. 2002), <0.1 to approximately 80 ng/L in Switzerland using a combination of reporter gene and gene expression assay (Rutishauser et al. 2004), 4 to 35 ng/L in Japan with a yeast reporter gene assay (Onda et al. 2002), <1 to 14.9 ng/L in Michigan (USA) using a mammalian reported gene assay (Snyder et al. 2001), and 21 to 147 ng/L EEq in Missouri (USA) with a yeast reporter gene assay (Tilton et al. 2002) have been reported. Using chemical separation techniques in a TIE approach, Desbrow et al. (1998) linked most of the estrogenic activity in municipal sewage effluent to the natural hormones 17β-estradiol ($E_2$) and estrone ($E_1$), the synthetic hormone ethinylestradiol ($EE_2$), and (to a lesser extent) the industrial surfactant nonylphenol (NP). The natural and synthetic hormones are from human excreta (Blok and Woesten 2000, Shore and Shemesh 2003). Nonylphenol is a derivative of alkylphenol polyethoxylates (APEOs) commonly used as nonionic surfactants in industrial processes (Johnson and Jürgens 2003). Studies have confirmed that these four chemicals ($E_2$, $E_1$, $EE_2$, and NP) contribute to most of the *in vitro* estrogenic activity in sewage in the UK (Sheahan et al. 2002b), The Netherlands (Belfroid et al. 1999), Germany (Körner et al. 2001, Spengler et al. 2001), Japan (Tamamoto et al. 2001), and the USA (Snyder et al. 2001). The levels of those four chemicals measured in effluents using chemical analytical methods (such as chromatography coupled with mass spectrometry) are from <1 to 64 ng/L for $E_2$, <1 to 82.1 ng/L for $E_1$, <1 to 62 ng/L for $EE_2$, and <1 to 330 µg/L for NP (reviewed in Christiansen et al. 2002). The concentrations of these chemicals in river water vary markedly, from <1 to 8.76 ng/L for $E_2$, <1 to 17 ng/L for $E_1$, <1 to 5.1 ng/L for $EE_2$, and <1 to 644 µg/L for NP

(reviewed in Christiansen et al. 2002). Most of the activity in water samples collected from two UK estuaries was due to $E_2$ and, to a lesser extent, NP (Thomas et al. 2001)

*In vivo* effects of EDCs in sewage have also been investigated. Laboratory exposure of fish to these chemicals at environmentally relevant concentrations result in estrogenic effects similar to those measured in the field. Adult male rainbow trout (*O. mykiss*) exposed to 10 ng/L of $E_2$ or 25 ng/L of $E_1$ for 3 weeks had significantly elevated plasma Vtg, as did male roach (*R. rutilus*) exposed to 100 ng/L of $E_2$ (Routledge et al. 1998). Juvenile rainbow trout exposed for 14 days to 10 ng/L of $E_2$, 32 ng/L of $E_1$, and 1.0 ng/L of $EE_2$ had significantly higher plasma vitellogenin than controls (Thorpe et al. 2003). Exposure of fathead minnows (*P. promelas*) to 100 ng/L of $E_2$ or 32 ng/L of $E_1$ for 21 days resulted in a significant increase in plasma Vtg, and exposure to 320 ng/L of E2 or E1 resulted in a significant decrease in gonadosomatic index (GSI; the weight of the gonads relative to body weight) (Panter et al. 1998). A significant increase in plasma Vtg was measured in male sheepshead minnows (*C. variegatus*) exposed to 100 ng/L of $E_2$ or $EE_2$ for 16 days (Folmar et al. 2000), and a significant Vtg mRNA induction was measurable after just 2 days of exposure (Denslow et al. 2001). Injections of NP likewise induced high levels of plasma Vtg in several species of fish (Christiansen et al. 1998a, Christiansen et al. 1998b, Christensen et al. 1999, Pait and Nelson 2003). Morphological and behavioral changes were also apparent in exposed fish, with early-life stages apparently the most sensitive (Hartley et al. 1998, van Aerle et al. 2002, Andersen et al. 2003b). For example, exposure of adult male and female zebrafish (*D. rerio*) to 10 ng/L of $EE_2$ for 24 days resulted in a reduction in GSI and abnormalities in gonadal histology (Van den Belt et al. 2002), but these changes were reversible after 24 days depuration. When Japanese medaka (*O. latipes*) were exposed from the time of hatching to about 80 - 120 days of age to 100 ng/L of $E_2$, $EE_2$, or $E_1$, a very high proportion of males exhibited ovotestis (Metcalfe et al. 2001). Exposure of Japanese medaka to 10 ng/L of $E_2$ for 28 days from hatch produced all females (Nimrod and Benson 1998). And mating pairs of Japanese medaka exposed to $E_2$ in their diet showed significantly reduced mating behaviour and fecundity (Oshima et al. 2003). Early-life stages may be particularly sensitive. While the estrogenicity of STP effluents appears to fluctuate seasonally (Kirk et al. 2002), intermittent exposure to these chemicals may be just as potent as continuous exposures (Panter et al. 2000).

Most studies on endocrine disruption in sewage have focused on compounds with estrogenic activity. However, androgenic hormones have also been found in river systems receiving STP effluents (Thomas et al. 2002), and may contribute to endocrine disruption. In the United

Kingdom for example, levels of <113 to 4000 ng/L dihydrotestosterone (DHT) equivalents (yeast assay, Kirk et al. 2002) and 34 - 635 ng/L DHT equivalents (yeast assay, Thomas et al. 2002) were reported in final effluents of several STPs, and male mosquitofish downstream of an STP discharge in Florida (USA) exhibited signs of exposure to androgenic chemicals (Angus et al. 2002).

In this thesis, a combination of bioassays was used to evaluate the estrogenic and androgenic potential of sewage at different levels of biological organization.

# 3. Development of methods for extraction and *in vitro* quantification of estrogenic and androgenic activity of wastewater samples

## 3.1. Abstract

Chemicals released into the environment by anthropogenic activities have been linked to estrogenic or androgenic effects in exposed wildlife. Environmental programs that monitor the activity in wastewater are currently handicapped by the cost and time required by cellular bioassays, and there is a need to develop and validate rapid and cost-effective methods to quantify the total estrogenic and androgenic activity of wastewater. In this study, estrogen receptors (ER) were isolated from sheep uteri and rainbow trout livers and androgen receptors (AR) were isolated from rainbow trout brains. The isolated receptors were used in competitive receptor binding assays to test the affinity of known estrogenic and androgenic chemicals for the receptor binding site, and results were compared with literature values for the rat uterine ER binding assay and the E-Screen. The relative binding affinities of the tested compounds to ER from different species were very similar, and binding to the ER was a more responsive endpoint than the cellular effect measured in the E-Screen. Using the sheep ER binding assay in combination with solid-phase extraction, the estrogenic activity in a raw sewage sample from a municipal treatment plant in Brisbane (Queensland, Australia) was measured at 51 – 73 ng/L estradiol equivalents (EEq).

## 3.2. Introduction

Chemicals released daily into the environment by anthropogenic activities can interfere with biological hormone signaling and homeostasis in a variety of organisms (WHO 2002, O'Connor and Chapin 2003). These chemicals, generally referred to as endocrine-disrupting compounds (EDCs), have been linked to developmental abnormalities and reproductive dysfunctions, particularly in aquatic animals (Guillette et al. 1994, Purdom et al. 1994, Depledge and Billinghurst 1999, Jobling and Tyler 2003, Matthiessen 2003). Most of these effects appear to be caused by an interference of the hypothalamo-pituitary-gonadal axis in

which estrogenic and/or androgenic hormones play a central role.

There is currently a need to validate rapid methods to evaluate the overall androgenic and estrogenic potential of different types of wastewater in order to develop risk assessment schemes (Fenner-Crisp et al. 2000, Huet 2000, ICCVAM 2003, Kinnberg 2003). In that context, chemical analyses have limitations in that they require a priori knowledge of the nature of the biologically active chemicals present in the sample and do not take into account the different potencies of chemicals or possible interactions among the multitude of chemicals present in complex mixtures. The most potent chemicals need only be present at trace concentrations to induce biological effects, and often are at or below analytical detection limits. In vitro bioassays, on the other hand, can provide an assessment of the overall biological activity in an environmental sample, making them ideal for rapid and large-scale screening. There are three main categories of *in vitro* bioassays available to assess estrogenic or androgenic activity of single compounds or complex mixtures, namely, competitive receptor binding assays, reporter gene assays, and cell proliferation assays (Kinnberg 2003, Körner et al. 2004). However, a validation of relationships between measurements at these different levels of biological signaling represented by these assays and the lack of ability to extrapolate to whole-organism responses makes it difficult to compare results between studies (Huet 2000, Ashby 2002, Safe et al. 2002). Receptor-binding assays, for example, are the cheapest but also the least specific of the three categories of *in vitro* bioassays, because they only assess the ability of a chemical to attach to the receptor-binding site. While binding is the initial step in the mechanism of action of steroid hormones (Danzo 1997), it does not necessarily imply activation or inhibition of the receptor-mediated cascade (Kinnberg 2003). Still, binding to the estrogen receptor is the major determinant or rate-limiting step in assays using living cells (Fang et al. 2000), and these molecular assays could therefore be used to determine the potential estrogenic and/or androgenic activity in complex mixtures. Cellular bioassays such as the MCF-7 breast cancer cell proliferation assay (E-Screen), which measure the potency of a sample to induce cell proliferation, also provide a measure of estrogenicity (Soto et al. 1995) and complement receptor-binding assays by providing a further dimension to the estrogenicity evaluation.

The purpose of this study was to compare a range of complementary bioassays to determine the estrogenic and androgenic activity in wastewater samples. Several known estrogenic and androgenic chemicals were tested with a sheep estrogen-receptor-binding assay, a rainbow trout estrogen-receptor-binding assay, an E-Screen, and a rainbow trout androgen-receptor-

binding assay. Results were compared with literature values for different species. The efficacy of three different solid-phase extraction (SPE) cartridges to extract estrogenic chemicals from a spiked sample and a complex raw sewage sample was also assessed.

## 3.3. Materials and Methods

### 3.3.1. Solid-phase extraction

Three reversed-phase SPE cartridges were tested: *a)* a Supelclean LC-18 (Supelco, part no. 505471) with 1 g of octadecyl-bonded endcapped silica sorbent in a 6-mL reservoir; *b)* an Oasis HLB (Waters Corp., part no. 186000115) with 0.5 g of an *n*-vinylpyrrolidone and divinylbenzene copolymer sorbent in a 6-mL reservoir; and *c)* an Isolute C2/C18(EC) (International Sorbent Technology, part no. 933-0100-C) with 1 g of a combination of C2 and C18 endcapped silica sorbent in a 6-mL reservoir.

All glassware was methanol-rinsed and dried prior to use. A 1-L sample of deionised water was spiked to 12 ng/L of 17β-estradiol ($E_2$, Sigma; predissolved in ethanol). Raw sewage samples were collected at a large municipal sewage treatment plant in Brisbane (Queensland, Australia) in 1-L glass bottles, brought back to the laboratory on ice, and processed immediately. The samples were centrifuged at 4500×*g* for 30 min at 4°C to remove large particulate matter, and 900 mL of the supernatant was decanted into a glass measuring-cylinder and then transferred to a glass flask. The SPE cartridges were preconditioned with 2×3 mL methanol followed by equilibration with 2×3 mL water, and the sewage and spiked samples were passed through the cartridges dropwise by vacuum suction (maximum of 70 kPa). After all of the 900-mL sample had passed, the cartridges were dried on the manifold for at least 10 min, capped, wrapped in aluminium foil, and stored at –20°C until elution.

The cartridges were eluted with 2×3 mL methanol. The eluate was evaporated at 50°C under gentle nitrogen stream, and reconstituted in 250 µL of methanol. The reconstituted samples were then evaluated using the bioassays.

### 3.3.2. Isolation of cytosolic estrogen receptors from sheep uteri

Estrogen receptors (ER) were isolated from sheep uteri based on protocols developed for rats (Shelby et al. 1996, EDSTAC 1998, ICCVAM 2003). All manipulations were carried out in a cold room at 4°C to minimize heat stress to the receptor proteins. The uteri were excised from freshly killed sheep at the abattoir and brought back to the laboratory in isotonic saline solution (0.9% NaCl) on ice. The tissue was trimmed of fat, snap-frozen in liquid nitrogen, and stored for up to one month at –80°C until further processing.

The uterine tissue was thawed in TEDG buffer (10 mM tris base, 2 mM EDTA, 10% glycerol, 1 mM dithiothreitol, pH 7.2 at 4°C), minced with a razor blade, and homogenized at 50 mg/mL in TEDG buffer with a polytron for three strokes of 3 s at 10,000 rpm, with care taken to minimize heating of the homogenizing probe. The homogenate was centrifuged at 1000×*g* for 10 min at 4°C. The supernatant was transferred to an ultracentrifuge tube and spun at 105,000×*g* for 50 min at 4°C. The final supernatant, containing cytosolic ER, was aliquoted and stored at -80°C until use.

### 3.3.3. Isolation of nuclear ER from rainbow trout livers

All fish manipulations were done pursuant to the New Zealand Animal Welfare Act (1999). The protocol to isolate ER from rainbow trout liver has already been described in detail elsewhere (Tremblay and Van Der Kraak 1998). Briefly, immature fish were anaesthetized with tricaine methanesulphonate (MS222, 0.1 g/L) and injected intra-peritoneally with $E_2$ (5 mg/kg, suspended in corn oil) weekly. After 3 weeks of induction, fish were anaesthetized in MS222 and euthanized by spinal severance. The liver was removed, washed in cold 0.9% NaCl and weighed. Livers of five males were then minced with a razor blade, pooled, and homogenized in three volumes of cold TED buffer (50 mM Tris-HCl, 1.5 mM $Na_2EDTA$, 1 mM dithiothreitol, 0.5 mM Pefabloc SC, pH 7.4 at 4°C) with three passes of a Teflon-pestle glass homogenizer. The homogenate was centrifuged at 1000×*g* for 20 min at 4°C. The pellet was washed twice with TED, and extracted in TED(KCl) (TED with 0.6 M KCl) at 1 g/mL for 1 h with occasional stirring. The extract was then centrifuged at 30,000×*g* for 30 min at 4°C. The final supernatant, containing the nuclear ER, was aliquoted and stored at -80 °C until use.

### 3.3.4. Isolation of nuclear androgen receptors from rainbow trout brains

The protocol to isolate androgen receptors (AR) from rainbow trout brains was adapted from Sperry and Thomas (1999a) and described in Bandelij (2003). Briefly, W buffer (50 mM Tris-HCl, 1.5 mM $Na_2EDTA$, 1 mM dithiothreitol, 30% glycerol, pH 7.5 at 4°C), H buffer (W buffer with 0.5 mM Pefabloc SC), and E buffer (H buffer with 0.7 M KCl) were prepared fresh. Adult male rainbow trout were anaesthetized with MS222 (0.1 g/L) and euthanized by spinal severance. Brains were excised and homogenized in two volumes of ice-cold H buffer with three passes of a Potter-Elvehjem Teflon-pestle homogenizer. The homogenates of approximately 70 fish were pooled and centrifuged at 2500×*g* for 15 min at 4°C. The pellet was washed twice with W buffer, and extracted in E buffer at 500 mg/mL for 1 h with occasional stirring. The extract was then centrifuged at 100,000×*g* for 60 min at 4°C. The final supernatant containing the nuclear AR was aliquoted and stored at -80 °C for later use.

### 3.3.5. Receptor binding assay

The pharmacokinetics of the receptor preparations for $E_2$ (in the case of ER) and testosterone (T; in the case of AR) were determined by saturation experiments (Scatchard 1949) and non-linear analysis of the resulting data (ICCVAM 2003), with the Langmuir equation, where $B_{max}$ is the total number of binding sites and $K_d$ is the dissociation constant:

$$\text{Langmuir equation: } y = \frac{B_{max} \times x}{K_d + x}$$

Both ER and AR competitive binding assays were adapted from the protocols in Shelby et al. (1996) and ICCVAM (2003). In brief, a serial dilution of the test chemical or sample (100 µL in the sheep ER, 50 µL in the rainbow trout ER and AR assays) was incubated with a known concentration of radio-labeled competitor for the receptor binding site (100 µL for 0.5 nM [2,4,6,7-$^3$H]$E_2$ in the sheep ER; 50 µL for 1 nM [2,4,6,7-$^3$H]$E_2$ in the rainbow trout ER; and 50 µL for 2.5 nM [1,2,6,7-$^3$H]T in the AR assay), a standard number of receptors (50 µL of ER for the sheep assay, 15 µL of ER or AR for the rainbow trout assays) and TEDG buffer (150 µL in the sheep ER, and 135 µL in the rainbow trout ER and AR assays), for a final incubation volume of 400 µL in the sheep ER and 250 µL in the rainbow trout ER and AR

assays. After 18 h at 4°C, dextran-coated charcoal (DCC; 0.5% w/v charcoal, 0.05% w/v dextran T70, in TEDG buffer) was added (200 µL in the sheep ER, 500 µL in the rainbow trout ER and AR assays) to strip any unbound radioligand from the supernatant. After a brief incubation at 4°C (12 min in the sheep ER, 5 min in the rainbow trout ER and AR assays), the tubes were centrifuged (1500×*g* for 12 min at 4°C in the sheep ER, 2000×*g* for 15 min in the rainbow trout ER and AR assays) and the supernatant (450 µL in the sheep ER, 550 µL in the rainbow trout ER and AR assays) was pipetted to a liquid scintillation vial. Liquid scintillation cocktail (2.5 mL Ultima Gold [Packard] in the sheep ER; 5 mL of scintillation cocktail [2 L toluene, 1 L Triton X-100, 12 g 2,5,-diphenyloxazole (PPO), 0.6 g 1,4-bis[2-5-phenyloxazolyl]benzene (POPOP)] in the rainbow trout ER and AR assays) was added and β radiation was measured by liquid scintillation (in a Wallac 1490 for the sheep ER assay; and a Packard Tri-Carb 2100-TR for the rainbow trout ER and AR assays). A displacement curve was obtained by plotting the proportion of radioligand still bound to the receptor against the test chemical concentration (or in the case of a water sample, against the equivalent volume tested). The $EC_{50}$, or effective concentration (or volume in the case of a water sample) required to displace 50% of the radioligand from the receptor binding sites, was calculated by fitting a Verhulst curve to the data by least-squares regression using an Excel9 module written by F. Leusch, where bottom and top refer to the minimum and the maximum $x$ value, respectively:

$$\text{Verhulst equation: } y = \frac{bottom + (top - bottom)}{1 + 10\text{^}[(\log EC_{50} - \log x) \times slope]}$$

The affinity of a test chemical for the receptor-binding site was assessed by determining its relative binding affinity (RBA), a ratio between the $EC_{50}$ of the standard ($E_2$ in the ER; T in the AR assay) and the $EC_{50}$ of the test chemical. The activity (estrogenic or androgenic) of a water sample was evaluated by calculating its estradiol- or testosterone-equivalent concentration (EEq or TEq, respectively), which is a ratio between the amount of the standard (in ng) in the incubation tube at $EC_{50}$ and the equivalent volume of the sample at $EC_{50}$ (in L).

### 3.3.6. MCF-7 cell proliferation assay (E-Screen)

The E-Screen assay was adapted from Körner et al. (1999) and Soto et al. (1995) with minor modifications. The breast cancer cell line (MCF-7) was a gift from R. Rosengren (University

of Otago, New Zealand) and originally sourced from the American Type Culture Collection (ATCC no. HTB-22). Cells were aliquoted in a 10% DMSO / 90% media solution at -80°C and cultivated in growth media consisting of phenol-red-free Dulbecco's modification of Eagle's medium (DMEM), supplemented with L-glutamine, fetal bovine serum (FBS), gentamycin and 2.5 M N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES), in an atmosphere with 5% $CO_2$ at 37°C. The steroid-free experimental medium consisted of phenol-red-free DMEM supplemented with dextran-coated charcoal-stripped FBS, 10 mM HEPES (1 M stock solution adjusted with 10N NaOH to pH 7.6), 2 mM L-glutamine, and gentamycin.

The MCF-7 cells were reconstituted in steroid-free experimental medium, and seeded at a concentration of 20,000 cells/mL in sterile 96 flat-bottom multi-well tissue culture plates (Nunclon). After 24 h, the medium was aspirated and replaced, and serial dilutions of test compounds added to the wells in eight replicates. On the sixth day (5 days after exposure), the assay was terminated, and cell proliferation in each well determined by analyzing protein content using a fluorescamine assay (Lorenzen and Kennedy 1993) in a fluorescence plate reader (FLUOStar model 403, BMG Lab Technologies). This colourimetric endpoint is, within a certain range, directly proportional to cell number (Körner et al. 1998).

Protein concentration in each set of wells was plotted against chemical concentration, and a Verhulst curve fitted by least-squares regression using an Excel9 module written by F. Leusch. The estrogenicity of the tested compounds was quantified with two parameters: *a)* the relative proliferative potency (RPP), which is the ratio of the lowest concentration of $E_2$ required for maximal cell yield divided by the lowest concentration of the tested compound required for maximal cell yield; and *b)* the relative proliferative effect (RPE), which is the ratio of the maximum cell yield obtained with the tested compound divided by the maximum cell yield obtained with $E_2$ (Soto et al. 1995). For statistical consistency, maximal cell yield was set at $EC_{95}$ (from the Verhulst curve), and the concentration of the chemical at $EC_{95}$ calculated as:

$$\log EC_{95} = \log EC_{50} + \left(\log \frac{95}{5}\right) \times \frac{1}{slope}$$

### 3.3.7. Statistical analyses

Data were analyzed with SPSS 10.0 (SPSS Inc., Chicago, IL, USA), with significance set at $\alpha$=0.05. Paired *t*-tests were used to compare the RBA of chemicals in the sheep ER assay with those obtained in a rat ER assay (from literature data) and the rainbow trout ER assay, and the RPP in the E-Screen. Paired *t*-tests were also used to compare RPE and RPP values in the E-Screen with values reported in the literature. When comparing these different endpoints, a linear regression was also used to determine if the data were correlated and to what extent. Analyses of variance (ANOVAs) were used to test for differences among the three different cartridges in EEq in the spiked samples, EEq in the raw sewage, and elution time of the raw sewage.

## 3.4. Results

### 3.4.1. Saturation experiments

The pharmacokinetics of the receptor preparations were determined by Scatchard assay. The number of $E_2$ binding sites ($B_{max}$) of the sheep ER preparations was 570 ± 150 fmol/mg of protein, and the dissociation constant ($K_d$) was 0.17 ± 0.01 nM (n = 3; Fig. 3.1A). The $B_{max}$ for the pooled rainbow trout hepatic ERs was 1.1 pmol/mg of protein, and the $K_d$ was 1.7 nM (Fig. 3.1B). With the pooled rainbow trout brain ARs, $B_{max}$ was 1.1 pmol/mg and the $K_d$ 0.41 nM (Fig. 3.1C).

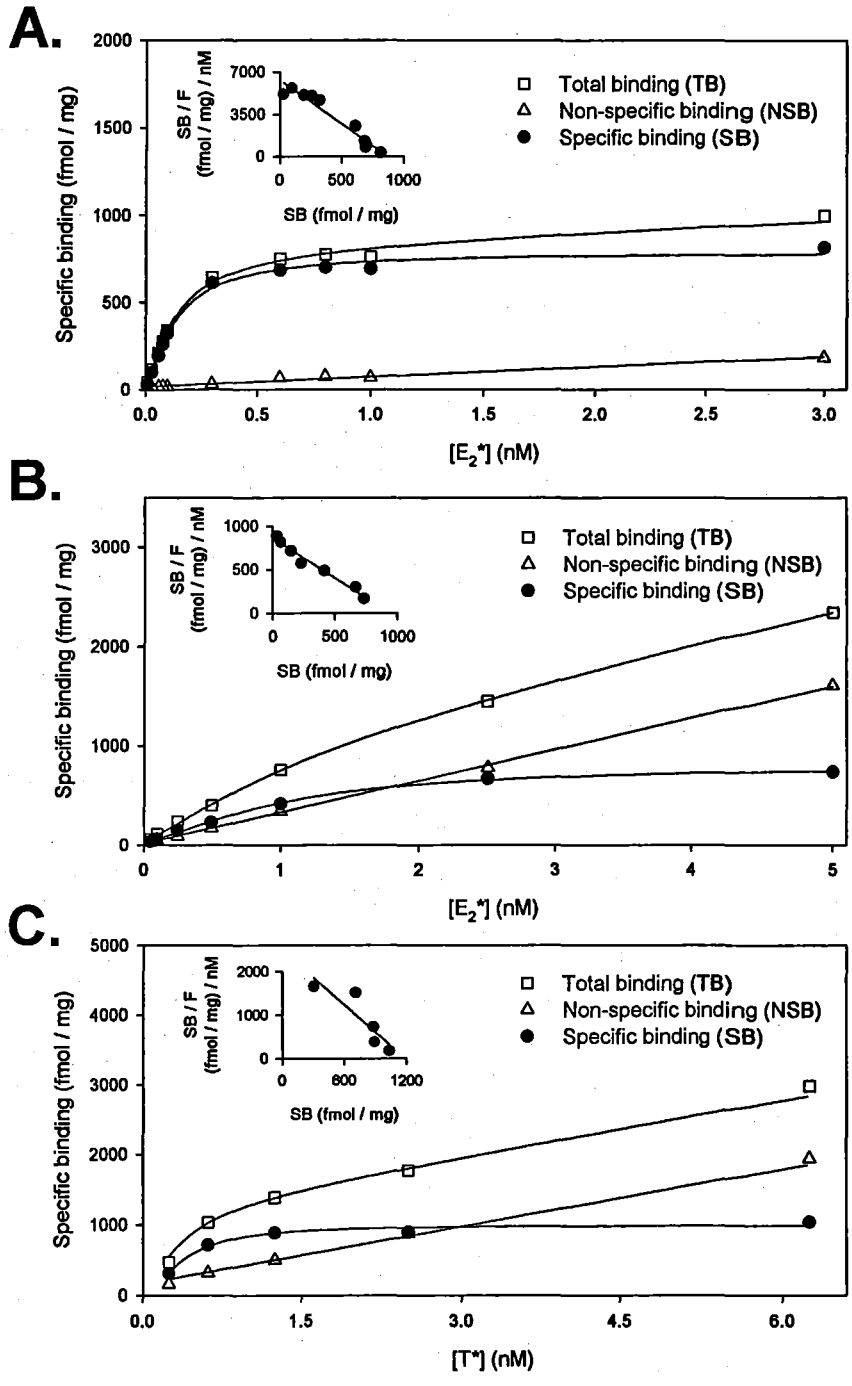


**Figure 3.1:** Saturation curves of receptor preparations. (A) Sheep uterine estrogen receptors. (B) Rainbow trout hepatic estrogen receptors. (C) Rainbow trout brain androgen receptors. (Inset) Scatchard plot.

### 3.4.2. Comparison of the bioassays using model compounds

The relative binding affinities (RBA) of several compounds for sheep and rainbow trout ER are reported in Table 3.1 (along with literature values for rat ER). There was a significant linear relationship between the affinity of the tested model compounds for the sheep ER and the rat ER ($n$=10, linear regression $P$<0.001, $R^2$=0.892, slope=0.898; Fig. 3.2A). On average, chemicals appeared to have a higher affinity (relative to $E_2$) for the rat ER, and RBA for the rat ER was on average 0.55 log units higher than for the sheep ER (paired $t$-test, $P$=0.009, $n$=10). Of the 18 chemicals tested in the rainbow trout ER assay, 11 caused a significant displacement of the radioligand from the receptor-binding site (Table 3.1). Only six different chemicals could be used for comparison with the sheep ER, and with this limited dataset there was a significant correlation in the affinity for the ER in both species ($n$=6, linear regression $P$=0.002, $R^2$=0.925, slope=1.279; Fig. 3.2B). The chemicals tested had a significantly higher affinity (relative to $E_2$) for the sheep ER than for the rainbow trout ER (paired $t$-test, $P$=0.015, $n$=6). The affinities of the tested chemicals for the rainbow trout AR are also reported in Table 3.1.

With the E-Screen (Table 3.2), there was a good linear relationship between the affinity of the chemical for the sheep ER and the proliferative potency (RPP) in the E-Screen ($n$=8, linear regression $P$=0.015, $R^2$=0.652, slope=0.955; Fig. 3.2C). RPP in the E-Screen were significantly lower than RBA in the sheep ER assay, by an average of 1.33 log units (paired $t$-test, $P$=0.014, $n$=8). When comparing E-Screen data to values reported in the literature (Fang et al., 2000; Körner et al., 2001), RPP values were very similar (paired $t$-test, $P$=0.317, $n$=7), and there was a clear linear correlation between the two datasets ($n$=7, linear regression $P$=0.004, $R^2$=0.835, slope=0.804; data not shown). With RPE values, there was no statistically significant difference between the two datasets (paired $t$-test, $P$=0.072, $n$=7), but there was also no linear relationship between the two ($n$=7, linear regression $P$=0.872, $R^2$=0.006).

**Table 3.1:** Relative affinity of tested chemicals for the sheep uterine estrogen receptor (ER), rainbow trout hepatic ER, and rainbow trout brain androgen receptor (AR) in this study, as well as values reported in the literature for rat uterine ER and Atlantic croaker brain AR.

| Compound | CAS RN | Estrogen receptor (ER) [1] | | | Androgen receptor (AR) [1] | |
|---|---|---|---|---|---|---|
| | | This study | | Literature[2] | This study | Literature[3] |
| | | Sheep ER (log RBA ± SE)[4] | Rainbow trout ER (log RBA) | Rat ER (log RBA) | Rainbow trout AR (log RBA) | Atlantic croaker AR (log RBA) |
| *Steroids* | | | | | | |
| 17β-Estradiol ($E_2$) | 50-28-2 | 0.00 | 0.00 | 0.00 | DND (-4.63) | DND |
| Testosterone (T) | 58-22-0 | < -4.60[5] | -4.94 | DND (-4.00) | 0.00 | 0.00 |
| Estrone (E1) | 53-16-7 | -0.84±0.09 | -1.37 | -0.43 | -5.02 | NA |
| Estriol (E3) | 50-27-1 | -0.12±0.13 | -1.49 | -0.68 | < -5.02[5] | NA |
| Progesterone | 57-83-0 | DND (-5.30) | DND (-6.16) | DND (-5.00) | -3.85 | -3.55 |
| Pregnenolone | 145-13-1 | < -5.30[5] | -5.75 | NA | DND (-4.54) | NA |
| Dihydrotestosterone | 521-18-6 | -3.82±0.05 | -5.63 | -2.77 | -0.83 | -1.20 |
| Dehydroepiandrosterone | 53-43-0 | NT | DND (-6.00) | -3.40 | -3.64 | NA |
| Epiandrosterone | 481-29-8 | NT | DND (-5.30) | NA | -2.51 | NA |
| Androsterone | 53-41-8 | NT | DND (-6.30) | NA | -1.47 | NA |
| Androstenediol | 521-17-5 | NT | NT | -1.22 | -2.99 | NA |
| Androstenedione | 63-05-8 | DND (-4.84) | DND (-6.00) | DND (-4.00) | 0.62 | DND |
| Androstadienedione | 897-06-3 | NT | DND (-6.00) | NA | 0.31 | NA |
| 11-Ketotestosterone | 564-35-2 | NT | NT | NA | -2.76 | -2.85 |
| 17-Methyltestosterone | 58-18-4 | NT | NT | NA | -1.60 | -1.55 |
| *Pharmaceuticals* | | | | | | |
| Ethinylestradiol ($EE_2$) | 57-63-6 | 0.29±0.09 | 0.81 | 0.94 | NT | NA |
| Mestranol | 72-33-3 | NT | -2.99 | NA | DND (-5.00) | NA |
| Tamoxifen | 10540-29-1 | -2.36±0.17 | -2.93 | -1.22 | -4.03 | NA |
| Flutamide | 13311-84-7 | NT | NT | NA | -4.37 | NA |
| Methyltrienolone | 965-93-5 | NT | NT | NA | -3.72 | NA |
| Cyproterone acetate | 427-51-0 | NT | NT | NA | -4.13 | NA |
| *Phytosterols* | | | | | | |
| β-Sitosterol | 83-46-5 | DND (-4.68) | DND (-4.87) | DND (-5.00) | < -5.09[5] | NA |
| Genistein | 446-72-0 | -0.84±0.12 | NT | -0.44 | NT | NA |
| Coumestrol | 479-13-0 | -0.95±0.06 | NT | 0.27 | NT | NA |
| Coprosterol | 360-68-9 | NT | -5.27 | NA | -4.41 | NA |
| *Pesticides* | | | | | | |
| *o,p'*-DDT | 789-02-6 | -3.52±0.01 | -4.52 | -3.05 | DND (-5.29) | DND |
| *p,p'*-DDT | 50-29-3 | DND (-5.85) | -5.27 | DND | DND (-5.29) | DND |
| *p,p'*-DDD | 72-54-8 | NT | NT | NA | DND (-5.29) | DND |
| *o,p'*-DDE | 3424-82-6 | NT | NT | NA | DND (-5.29) | DND |
| *Industrial chemicals* | | | | | | |
| *p*-Nonylphenol | 84852-15-3 | -4.13±0.24 | NT | -3.51 | DND (-5.59) | NA |
| 4-*t*-Octylphenol | 140-66-9 | -3.47±0.04 | NT | NA | NT | NA |
| Bisphenol A | 80-05-7 | -2.64±0.11 | NT | -2.48 | NT | NA |

CAS RN = Chemical abstracts registry number; NT = Not tested; NA = Data not available; DND = Chemical did not displace more than 20% of the radioligand from the receptor binding site at the highest concentration tested. The chemical may displace the radioligand at higher concentrations, and the equivalent RBA at the highest concentration tested is therefore given in brackets.

[1] Relative binding affinity (RBA) expressed relative to 17β-estradiol in the ER and to testosterone in the AR.

[2] Values for ER binding assay are from rat ERβ data in Kuiper et al. (1997) and Fang et al. (2000).

[3] Values are from Atlantic croaker AR1 data in Sperry and Thomas (1999a, 1999b).

[4] Calculated over several assays with receptors from two different sheep, except for tamoxifen and *p*-nonylphenol, which are from three.

[5] Slight displacement (more than 20%, but less then 50%) at the highest concentration tested, insufficient to calculate an accurate $EC_{50}$.

**Table 3.2:** Relative proliferative effect (RPE) and potency (RPP) of selected chemicals relative to 17β-estradiol in the MCF-7 cell proliferation assay (E-Screen) compared with values reported in the literature (Fang et al. 2000, Körner et al. 2001).

| Compound | CAS RN | RPE (%) ± SE | | log RPP ± SE | |
|---|---|---|---|---|---|
| | | This study[1] | Literature[2] | This study[1] | Literature[2] |
| *Steroids* | | | | | |
| 17β-Estradiol ($E_2$) | 50-28-2 | 100 | 100 | 0.00 | 0.00 |
| Estrone (E1) | 53-16-7 | 77 ± 5 | 104 ± 8 | -1.91 ± 0.44 | -1.19 ± 0.17 |
| Dihydrotestosterone (DHT) | 521-18-6 | 35 ± 29 | 102[3] | -6.40 ± 1.37 | -4.37[3] |
| Androstenedione | 63-05-8 | DND | NA | DND | NA |
| *Pharmaceuticals* | | | | | |
| Ethinylestradiol ($EE_2$) | 57-63-6 | 74 ± 26 | 98 ± 6 | 0.03 ± 0.25 | 0.01 ± 0.05 |
| *Phytosterols* | | | | | |
| β-Sitosterol | 83-46-5 | DND | DND[4] | DND | DND[4] |
| Genistein | 446-72-0 | 98 ± 50 | 115 ± 8 | -3.81 ± 1.12 | -3.70 ± 0.15 |
| Coumestrol | 479-13-0 | 150 ± 127 | NA | -2.30 ± 0.38 | NA |
| *Industrial compounds* | | | | | |
| *p*-Nonylphenol | 84852-15-3 | 46 | 98 ± 6 | -4.11 | -4.33 ± 0.21 |
| 4-*t*-Octylphenol | 140-66-9 | 62 ± 2 | 89 ± 7 | -3.64 ± 0.05 | -4.19 ± 0.07 |
| Bisphenol A | 80-05-7 | 126 ± 107 | 93 ± 4 | -4.90 ± 0.25 | -4.53 ± 0.25 |

CAS RN = Chemical abstracts registry number; DND = Chemical did not displace (RPE <15%); NA = Data not available.

[1] Value is the mean±SE of two assays on two separate occasions except for 4-nonylphenol, which is for only one assay.

[2] Value is the mean±SE of values reported in Fang et al. (2000) and Körner et al. (2001), except when marked with [3] when it is from Fang et al. (2000) only, or with [4] when it is from Körner et al. (2001) only.

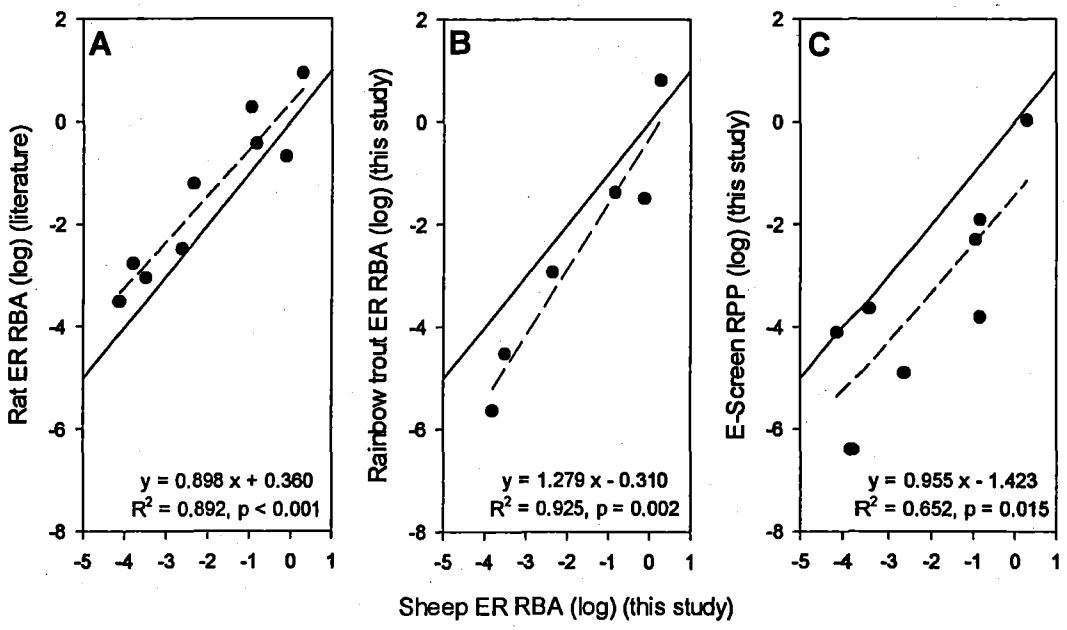


**Figure 3.2:** Comparison of relative binding affinities to the sheep uterine estrogen receptor (ER) on the abscissa vs. (A) the rat ER, data from Kuiper et al. (1997) and Fang et al. (2000); (B) the rainbow trout ER; and (C) the relative proliferative potency (RPP) in the E-Screen on the ordinate. The unbroken line is the isometric line. The equation, $R^2$, and $p$-value of the best regression line (dashed line) are at the bottom right of each graph.

### 3.4.3. SPE retention efficiency and raw sewage

There was no statistically significant ($P$>0.05) difference in recovery of the $E_2$ spike among the different cartridges (one-way ANOVA, $P$=0.076), although the Oasis HLB was the only one with a recovery higher than 90% (Table 3.3). When eluting a raw sewage sample, there were no significant differences in EEq or elution time among the different cartridges (one-way ANOVA, $P$=0.477 and $P$=0.084, respectively) (Table 3.3). The total estrogenic activity in the raw sewage sample measured with the sheep ER binding assay was 51 – 73 ng/L EEq (Table 3.3). An unidentified cloudy substance eluted with the LC-18 cartridge, possibly a leachate from the sorbent bed. It did notappear to have an impact on estrogenicity of the sample, however, as indicated by the relatively similar EEq calculated with the different cartridges.

**Table 3.3:** Estradiol equivalents (EEq; determined by sheep estrogen receptor binding assay) for spiked solid-phase extraction (SPE) cartridges (spiked with 12 ng/L 17β-estradiol) and raw sewage samples. Also shows the recovery efficiency of the spike, and elution time of the raw sewage sample. Values are the mean ± SE of two separate SPE cartridges.

| SPE cartridge | 17β-estradiol spike | | Raw sewage sample | |
|---|---|---|---|---|
| | EEq (ng/L) | % recovery | EEq (ng/L) | Elution time (h) |
| Supelclean LC-18 | 9.39 ± 0.15 | 77.4 | 51.0 ± 3.46 | 9.3 ± 1.1 |
| Oasis HLB | 11.1 ± 0.42 | 92.5 | 64.2 ± 9.30 | 5.2 ± 0.6 |
| C2/C18(EC) | 10.4 ± 0.35 | 86.2 | 72.6 ± 16.5 | 6.6 ± 0.7 |

## 3.5. Discussion

In this study, the potencies of known estrogenic chemicals evaluated by a sheep uterine ER binding assay were well correlated with those obtained with rainbow trout hepatic ER and literature values for rat uterine ER, as well as with a cellular response measured by the E-Screen (Fig. 3.2).

The dissociation equilibriums ($K_d$) for $E_2$ with the rainbow trout hepatic ER preparations were similar to those reported in the literature (Tremblay and Van Der Kraak 1998), but the total number of binding sites per milligram of protein ($B_{max}$) was about 6× higher in this study. This is likely to be a reflection of the longer induction period in this study (3 weeks) compared with Tremblay and Van Der Kraak (1998) (6 days). When compared with hepatic estrogen receptors isolated from other teleosts such as the common carp, the rainbow trout receptors in this study had a very similar $K_d$ (1.7 vs. 1.4 – 2.1 nM) but a much higher $B_{max}$ (1.1 vs. 0.02 – 0.03 pmol/mg in carp) (Kloas et al. 2000). This may again be due to the long induction time, but also probably reflects a higher sensitivity of rainbow trout to estrogenic stimulation. There were many similarities between the sheep uterine ER and values for the rat uterine ER reported in the literature. For example, the $B_{max}$ and $K_d$ for $E_2$ with the sheep uterine ER preparations were very similar to those reported for rat uterine cytosolic ERs (Boctor et al. 1983, Levin et al. 1993).

The chemicals tested had a slightly higher affinity for the rat ER than for the sheep ER (Fig. 3.2A, Table 3.1) and, in turn, a slightly higher affinity for the sheep ER than for the rainbow trout ER (Fig. 3.2B, Table 1). There was, however, a significant linear relationship in

affinities between binding to ER from sheep and from the other two species (Fig. 3.2A and 3.2B) and a favorable agreement in negative results (DND, Table 3.1). This suggests that the affinity of a chemical for the rat or rainbow trout ER could be predicted from its affinity for the sheep ER. The sheep ER binding assay has a number of ethical and financial advantages over the rat or rainbow trout assays. A large volume of samples can be analyzed with receptors isolated from one sheep uterus for example, because of its larger size. This minimizes the number of animals sacrificed. Uteri can be collected at local abattoirs, removing the expense associated with animal husbandry. The sheep ER assay thus seems an ideal alternative to the rat or rainbow trout ER assays.

With the rainbow trout brain AR assay, $B_{max}$ was similar to that previously reported for AR isolated from rainbow trout brains (1.1 vs. 0.9 pmol/mg) (Wells and Van Der Kraak 2000). The $K_d$ was slightly lower (0.41 vs. 1.43 nM), however, indicating a higher-affinity preparation in this study. This may be due to differences in reproductive state at sampling. There are few data available in the literature describing the affinity of chemicals for AR. Too few compounds were available for a statistically rigorous comparison between this assay and the one reported in Sperry and Thomas (1999a, 1999b). There was a generally good agreement for those chemicals that were available for both datasets, namely dihydrotestosterone, progesterone, 11-ketotestosterone, and 17-methyltestosterone, and with chemicals that did not displace in either study (e.g., organochlorines and $E_2$) (Table 3.1). This suggests that the chemicals would have similar affinities for AR from both species, with some exceptions (as appears to be the case with androstenedione, Table 3.1). Despite repeated efforts, we were unable to obtain a high-specificity cytosolic or nuclear androgen receptor preparation from male sheep reproductive tissue (epididymis, bulbo-urethral gland, vas deferens, or seminal vesicle; data not shown) using the protocols developed for rats (EDSTAC 1998, ICCVAM 2003), and affinity of chemicals to AR from both species could thus not be compared.

The RPP values for the E-Screen, which estimate the potency of the tested chemicals to induce an estrogenic effect, were very similar to those reported in the literature (Table 3.2); however, the RPE values, which estimate the amplitude of the induced effect, were not (Table 3.2). This was not surprising in light of the multitude of differences in E-Screen protocols between laboratories (such as differences in sourcing of reagents and cell stocks), which can have a tremendous effect on the amplitude of the estrogenic response (Villalobos et al. 1995). There was also a large amount of variation in RPE results, which illustrates the inherent

variability of assays of higher biological complexity such as the E-Screen. Overall, however, the molecular response seen in the sheep ER assay was well correlated with the response measured in the E-Screen (Fig. 3.2C). This suggests that binding to the ER is a good predictor of the whole cellular response, as has been previously suggested (Fang et al. 2000). Quantitatively, the affinity of the tested chemicals (RBA) for the sheep ER binding site was on average 21× higher than their ability to induce cell proliferation (RPP) in the E-Screen (Fig. 3.2C). There was also a wider spread of the data around the line of best regression between the two assays ($R^2$=0.652, Fig. 3.2C), which was indicative of the type of effect (agonistic or antagonistic) resulting from binding to the receptor. For example, $EE_2$, OP, and NP (the three points closest to the isometric line) have a clearly agonistic effect on cell proliferation after binding to the ER. In contrast, bisphenol A and genistein appear to have only a weakly agonistic activity, despite their good affinity for the ER binding site (as indicated by their much lower RPP than RBA, relative to $E_2$). This highlights one of the advantages of a receptor-binding assay, which identifies chemicals that interfere with the basic mechanism of the receptor-mediated cellular response. Binding to the receptor in itself is an indicator that the chemical is likely to be of concern, as sequestration of the receptor from the pool of available receptors for a normal endocrine response may have consequences *in vivo*.

It may be possible to roughly predict the affinity of a chemical for receptors from different species based on its affinity for the receptor in one standard species. For example, if a chemical had an RBA of 0.1 for sheep uterine ER, one would predict it to have an RBA of 0.29 for rat uterine ER and 0.026 in the rainbow trout hepatic ER based on the equations in Fig. 3.2. One should be mindful, however, not to assume that this good correlation in binding affinities implies a subsequent correlation of effects at the organism level. While estrogenic chemicals may bind to ER from different species with very similar affinities (Matthews et al. 2000), there can be wide differences in the magnitude of the induced gene expression between different species (Matthews et al. 2002). This illustrates one of the most significant limitations of receptor-binding assays. A positive result in a receptor-binding assay does not necessarily translate into receptor activation and subsequent *in vivo* effects, but rather indicates the potential for such an effect and warrants further confirmatory testing in more complex *in vitro* and particularly *in vivo* assays. Other studies have also shown that different isoforms of the receptors within the same species can have different binding characteristics (Kuiper et al. 1997, Sperry and Thomas 1999a), and care has to be taken when comparing

receptors from different tissues. The sheep ER binding affinities reported in this study are most likely for the ERβ, the dominant isoform in uterine tissue (Kuiper et al. 1997). Receptor binding assays also generally lack a metabolic activation step, which may cause false negatives for chemicals that are not themselves biologically active but have metabolites that are (such as methoxychlor; Shelby et al. 1996). And while binding to the steroid receptor is the first step in a receptor-mediated response, *in vivo* responses can be mediated via other pathways (Zacharewski 1998), and a battery of assays including *in vivo* assays should be considered in any comprehensive monitoring program on endocrine disruption.

However, despite all these limitations, receptor-binding assays still provide a reliable and cost-effective platform for large-scale screening of chemicals for estrogenic or androgenic activity. Binding to the receptor is a clear indication that the chemical in question has the potential to hijack the hormonal machinery of the organism and suggests the need for further *in vivo* screening. Combined with solid-phase extraction methods, receptor-binding assays would allow rapid screening of complex water samples such as sewage or industrial wastewater for a *potential* to induce estrogenic and androgenic effects *in vivo*. For example, raw sewage extracts from Brisbane (Queensland, Australia) tested with the sheep ER binding assay in this study revealed that it contained a significant estrogenic activity, with levels similar to those reported by researchers in Europe and Japan (Körner et al. 2000, Murk et al. 2002, Onda et al. 2002). A survey to determine the estrogenic and androgenic activity of treated sewage at several treatment plants is now underway to determine the potential risks associated with discharge of treated sewage in Queensland.

## 3.6. Co-author contributions

This chapter has been submitted to the journal Comparative Physiology and Biochemistry Part C. Besides supervisory committee members, co-authors for this paper are Anna Eriksson (Landcare Research, Lincoln, New Zealand), and Mike van den Heuvel (Forest Research, Rotorua, New Zealand). Anna developed and carried out the E-Screen assay at Landcare Research. Mike provided logistical support for the work with the AR binding assay, including the necessary facilities and supplies, as well as access to the AR isolation protocol.

# 4. Efficacy of an advanced sewage treatment plant in south-east Queensland (Australia) to remove estrogenic chemicals

## 4.1. Abstract

The estrogenicity profile of domestic sewage during treatment at a medium-sized (3800 EP) advanced biological nutrient removal plant in Queensland (Australia) was characterised using a sheep estrogen receptor binding assay (ERBA) and the MCF-7 breast cancer cell proliferation assay (E-Screen). The raw influent was highly estrogenic (20 - 54 ng/L EEq), and primary treatment resulted in a slight increase in estrogenicity that was detected in one of the assays (6 - 80 ng/L). Concurrent chemical analysis suggested that most of the estrogenicity in the influent was due to natural hormones (>48%). Secondary activated sludge treatment followed by nitrification/denitrification effectively removed >95% of the estrogenic activity (to <0.75 - 2.6 ng/L), and estrogenicity of the final tertiary-treated effluent was below the detection limit of both assays (<0.75 ng/L).

## 4.2. Introduction

Fish exposed to treated sewage have been shown to exhibit reproductive abnormalities consistent with estrogenic endocrine disruption (Jobling and Tyler 2003). Several studies have identified the natural steroids 17β-estradiol ($E_2$), estrone ($E_1$), and the synthetic estrogen ethinylestradiol ($EE_2$) as the most potent estrogenic compounds in treated municipal sewage (Desbrow et al. 1998, Snyder et al. 2001). Laboratory exposures have confirmed these chemicals to be estrogenic to fish *in vivo* at very low (ppt) concentrations, with effects similar to those observed in the field (Foran et al. 2000, Metcalfe et al. 2001, Tabata et al. 2001, Folmar et al. 2002). The main source of these chemicals in domestic sewage is from human waste (Blok and Woesten 2000, Shore and Shemesh 2003). As hormones are naturally excreted, the focus of a strategy to manage the potential environmental impact of sewage must shift to identifying and implementing effective treatment technologies to remove these chemicals effectively before discharge into the environment.

Studies have shown that secondary treatment of sewage, and in particular activated sludge

treatment, is very effective at removing estrogens (Johnson et al. 2000, Matsui et al. 2000, Nasu et al. 2001, Kirk et al. 2002, Svenson et al. 2003). A German municipal sewage treatment plant (STP) removed over 98% of the natural estrogens ($E_1$ and $E_2$) and more than 90% of $EE_2$, mostly during activated sludge treatment (Andersen et al. 2003a). In experiments with activated sludge from STPs, $E_2$ was quickly converted into $E_1$, which was then slowly degraded (Ternes et al. 1999a, Onda et al. 2003). In STPs in the USA, Layton *et al.* (2000) showed that 70 - 80% of $E_2$ was degraded within 24h through mineralization and 10 - 20% was sorbed to biosolids, for a total removal of $E_2$ from the aqueous phase of over 90%. However, in aerobic batch experiments with activated sludge, $EE_2$ was not significantly degraded after 48h (Ternes et al. 1999a). Only a small fraction (20%) was degraded by activated sludge, but removal from the aqueous phase was still about 80% due to high sorption to the biosludge (Layton et al. 2000). Joss et al. (2004) reported significant and rapid degradation of $E_2$ and $E_1$ in both anaerobic and aerobic conditions (albeit at a faster rate in the latter), while $EE_2$ was only significantly degraded in aerobic conditions.

Due to the broad socioeconomic differences between Australia and Europe or North America, data generated abroad must be confirmed locally. In 2004 for example, agriculture was a more important part of the economic landscape in Australia (3.5% of the GDP) than in the United Kingdom (0.9%), Germany (1%), the United States (1.4%), or even France (2.7%) (CIA 2004). Social factors can also have an impact on the presence of hormones in the environment, with much higher use of the pill as a form of contraceptive by women of reproductive age in Western Europe (48.2%) than in North America (15.5%) or Australia (24.0%) (UN 2003). There are also climatic differences, for example the monthly average temperature in Brisbane in the four seasons prior to sampling was similar to that of Houston (Texas), and much warmer than other selected European and North American cities (NCDC 2000). Layton *et al.* (2000) showed that temperature can significantly affect the rate of degradation of $E_2$ and $EE_2$ in activated sludge, and these differences could significantly affect the presence and degradation of hormones in sewage in Queensland, Australia. To the authors' knowledge, there are to date no other studies on the estrogenicity profile of sewage during treatment in Australia. The present study used chemical extraction methods followed by two different bioassays to examine the estrogenic profile of sewage along the treatment train at an advanced STP with an activated sludge system in subtropical Australia. The main purpose was to determine the efficacy of each step of the treatment train at removing estrogenic compounds.

## 4.3. Experimental

### 4.3.1. Site description.

The Landsborough STP operated by CalAqua (Caloundra City Council) treats approximately 0.98 ML of raw sewage per day (3800 equivalent people) from the town of Landsborough in south-east Queensland, Australia, >95% of which is from domestic sources. It is an advanced tertiary treatment facility originally designed for biological and nutrient removal (BNR), and has been the focus of a recent study where a suite of suspected EDCs (such as pesticides, herbicides, PCBs, lead, mercury, cadmium, $E_2$, $E_1$, and $EE_2$) were measured by chemical analysis (Chapman 2003). Primary treatment consists of a screen and a grit and grease chamber (Fig. 4.1). Secondary treatment includes anaerobic selectors, a large sequencing batch reactor (bioreactor) followed by nitrification/denitrification, and a secondary clarifier. The sludge is returned from the secondary clarifier to the anaerobic selectors, while the aqueous phase flows to tertiary treatment. Average sludge retention time at the time of sampling was 25.8 days. Tertiary treatment includes sand filters, ozone contact tanks, bioactivated carbon filters, and UV disinfection banks.

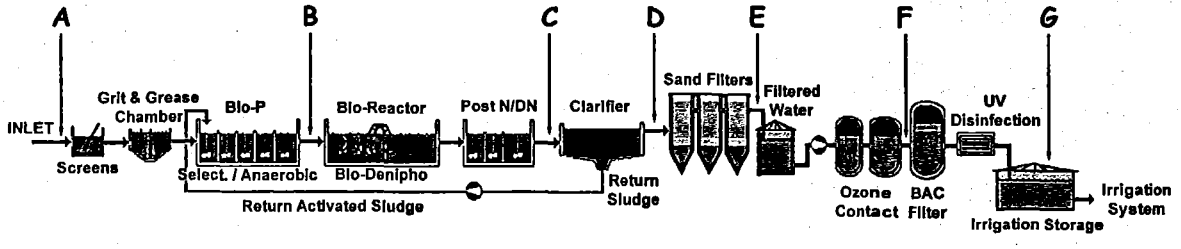


**Figure 4.1:** Treatment train at the Landsborough sewage treatment plant, including sample collection points: A) Raw; B) Post-anaerobic selectors; C) Post-nitrification/denitrification; D) Final-secondary-treated; E) Post-sand filtration; F) Post-ozonation; G) Final tertiary-treated.

### 4.3.2. Sample collection and extraction

Samples were collected at seven different stages of the sewage treatment train (Fig. 4.1, labeled A through G) on 21 August 2000 at 0900h. In the 48h prior to sampling, there was no

rain in the sampling area and the minimum and maximum air temperature were 9 – 25°C (data courtesy of the Commonwealth Bureau of Meteorology). The water temperature at time of sampling was 19°C. Duplicates of each sample were collected in methanol-rinsed 1 L glass Schott bottles and kept on ice until extraction within 24h of collection. The solid-phase extraction (SPE) protocol was based on the method of Lee and Peart (1998). Before extraction, each sample was vacuum filtered through a Whatmann filter with a pore size of 1.2 µm. Polypropylene cartridges with 1g of end-capped octadecyl reversed-phase sorbent and 6 mL reservoir (International Sorbent Technology no. 221-0100-C) were used to extract organic contaminants from the aqueous phase. The cartridges were loaded on a SPE manifold and pre-conditioned with 5 mL acetone, 5 mL methanol, and 10 mL double-distilled water. The filtered sample (1 L) was applied to the SPE cartridge under –70 kPa vacuum, at approximately 10 mL/min. When extraction was complete, the cartridges were dried on the manifold for 5 min, rinsed with 2×5 mL acetone/water (1:4, v/v), wrapped in aluminum foil, and stored at -20°C. Recovery efficiency for $E_2$, determined by spiking 1 L of water with 12 ng of $E_2$, was 75%. Blanks were made from distilled water and put through the same manipulation as the samples.

### 4.3.3. Estrogen receptor binding assay

For analysis in the ER binding assay, each SPE cartridge was eluted with 2x2.5 mL acetone (polar fraction), 2x2.5 mL diethylether (mid-polar fraction), and 2x2.5 mL *n*-hexane (non-polar fraction), under low vacuum. The solvent in each fraction was evaporated under gentle nitrogen stream, and the samples reconstituted in 100 µL dimethylsulfoxide (DMSO).

The ER binding assay protocol has been described previously (Tremblay et al. 2004). Briefly, uteri were excised from adult ewes, trimmed of fat, and homogenized in TEDG buffer (10 mM Tris, 2 mM EDTA, 10% glycerol, pH to 7.2 at 4°C; 1 mM dithiothreitol immediately before use). The homogenate was centrifuged at 1,000×*g* for 10 min at 4°C. The pellet was discarded and the supernatant centrifuged at 105,000×*g* for 50 min at 4°C. The final supernatant, containing the unoccupied cytosolic receptors, was aliquoted into 2 mL polypropelene centrifuge tubes and snap-frozen in liquid nitrogen. The aliquots were stored at –80°C. Typical results for sheep ER exhibited kinetics linked to limited capacity ($B_{max}$ = 570 ± 150 fmol/mg) and high affinity ($K_d$ = 0.17 ± 0.01 nM) (Chapter 3). For the ER binding assay, serial dilutions of the samples were incubated with a known concentration of

radiolabelled 17β-estradiol ([2,4,6,7-$^3$H]$E_2$; $^3HE_2$; Amersham Pharmacia Biotech NZ, Auckland, New Zealand) and a fixed amount of receptors (standardized between preparations by the number of binding sites $B_{max}$) for 18h at 4°C. At the end of the incubation period, free $^3HE_2$ was stripped from the incubation medium by addition of dextran-coated charcoal (DCC; 0.5% w/v charcoal, 0.05% w/v dextan T70, in TEDG buffer) and the remaining bound $^3HE_2$ measured by liquid scintillation. The $EC_{50}$ (or in the case of a water sample the $EV_{50}$, the equivalent volume of sample needed to displace $^3HE_2$ from half the receptor binding sites) was determined by least square regression of a Verhulst curve with a VBA6 module for Excel9 written by F. Leusch.

$$\text{Verhulst equation: } y = \frac{bottom + (top - bottom)}{1 + 10\text{^}[(\log EC_{50} - \log x) \times slope]}$$

The estrogenicity of the samples was expressed as estradiol equivalents (EEq), or the equivalent concentration of $E_2$ that would have to be present in the sample to achieve a response of the same amplitude. EEq is calculated as the ratio between the amount of $E_2$ in the incubation tube at $EC_{50}$ in the standard curve and the equivalent volume at $EC_{50}$ ($EV_{50}$). With 1 L of sample, the method detection limit (MDL) was 0.75 ng/L.

### 4.3.4. MCF-7 breast cancer cell proliferation assay (E-Screen)

For the E-Screen, organics were eluted from the cartridges with 2x2.5 mL acetone. For samples A and D, a second elution was performed with 2x2.5 mL ethyl acetate and analysed separately in the E-Screen assay to check for completeness of elution. For sample A, 5.5% of the estrogenic activity of the first eluate (expressed in EEq) was found in the second eluate, while no estrogenicity was detectable in the second eluate of sample D. Fifty microliters of DMSO were added to each extract and the solvent evaporated completely under a gentle stream of nitrogen. Stock solutions of the extracts were prepared with steroid-free experimental medium [phenol red-free Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% charcoal-dextran treated fetal calf serum (CD-FCS), 10 mM HEPES buffer, 2 mM L-glutamine, 1% of a solution of non-essential amino acids (NEAA) and 1% of a penicillin/streptomycin/amphotericin solution]. The preparation of the medium is described in detail elsewhere (Körner et al. 1999). Experimental medium (4.95 mL) was added to each

sample, homogenized for 1 min and the clear solution filtered sterile through a 0.22 µm Millex-GS filter (Millipore, France). These stock solutions containing 1% (v/v) DMSO were stored in sterile 5 ml glass flasks at 4°C. For cell culture testing, aliquots of the stocks were diluted 10- to 2000-fold (0.05 to 10 L final volume assuming that the whole extract is diluted) with steroid-free experimental medium using sterile 15ml polypropylene vials (Sarstedt, Germany). The maximum solvent concentration in the culture medium did not exceed 0.1%, a concentration that did not affect cell proliferation.

The assay was based on Körner et al. (2001) with minor modifications. Estrogen receptor-positive human MCF-7 breast cancer cells were cultivated in 25 $cm^2$ flasks (Sarstedt) in DMEM with 15 mg/L phenol red and 2 mM N-acetyl-L-alanyl-L-glutamine (Biochrom, Germany) at 37°C in a water-saturated atmosphere of 5% $CO_2$/ 95% air. The culture medium was supplemented with 5% FCS, 1% of NEAA and 1% of penicillin/streptomycin/amphotericin. Subconfluent MCF-7 cells were trypsinized, washed with culture medium, and resuspendend in steroid-free experimental medium.

Cells were seeded into 96-well plates (Sarstedt) in 75 µl of experimental medium at a density of 1500 cells per well. After 24h, 75 µl of experimental medium was added to each well, containing a series of dilutions of the stock solutions of sewage extracts. Each dilution was tested in eight replicates per assay. Eight wells per assay without hormones acted as negative control. $E_2$ in five final concentrations between $10^{-12}$M and $10^{-9}$M was the internal positive control in each assay. Previous studies have shown that higher E2 concentrations do not induce a higher proliferative response (Körner et al. 1999). Five days later (*i.e.* day 6) the assay was terminated during the late exponential phase of proliferation, and the cell number in each well was determined by measuring total protein content using the sulforhodamine B (SRB) assay (Skehan et al. 1990). In brief, cells were fixed with cold 10% (w/v) trichloroacetic acid, stained with 0.4% (w/v) solution of sulforhodamine B in 1% acetic acid, washed and dried. The dye was dissolved in 100 µl cold 10 mM Tris buffer (pH 10.5) per well and the extinction at 550 nm (reference 630 nm) measured in a microplate reader (Dynex MR 1200). The extinction of SRB at 550 nm is directly proportional to the cell number within a wide range (Skehan et al. 1990, Körner et al. 1998).

The endpoint of the E-Screen assay is the cell number relative to the hormone free control. The proliferative effect (PE) of a sample is the ratio of the highest cell number achieved with the sample to the cell number of the negative control. The estrogenic activities of sewage

samples were evaluated quantitatively by determining the relative proliferative effect (RPE) and the estradiol equivalent concentration (EEq). RPE is a measure of relative estrogenic efficacy and compares the maximum proliferation induced by a sample with that induced by the positive control $E_2$ at 1nM. This enables the distinction of full agonistic activity (RPE = 80 - 100%) from partial agonistic activity (RPE <80%) (Soto et al. 1995). The EEq is the total amount of estrogenic active compounds in a sample normalized to $E_2$, and is computed as described above. EEq values for sewage samples were calculated irrespective of whether a full response was obtained or not. PE, RPE, $EC_{50}$, and EEq values of sewage samples were calculated for each individual experiment. The log-probit regression analysis and calculation of $EC_{50}$ values were done with a VBA module for Excel5 written by Josef Greve (Fraunhofer Institute of Environmental Chemistry and Ecotoxicology, Schmallenberg, Germany).

### 4.3.5. Chemical analysis (GC/MS)

GC/MS analyses were carried out by the Australian Government Analytical Laboratory (AGAL; Pymble, NSW, Australia). Analytical methods to measure the levels of $E_2$, $E_1$, and $EE_2$ were developed by AGAL based on the methods of Lee and Peart (1998). Briefly, samples were extracted by SPE as described above, derivatised with PFPA followed by GC/MS-SIM analysis. The limit of reporting was 5 ng/L for all three chemicals.

## 4.4. Results

### 4.4.1. Simple fractionation

In the ER binding assay, none of the mid-polar and non-polar eluates (eluted with diethylether and n-hexane, respectively) significantly displaced $E_2$ from the ER binding site (data not shown). All the estrogenic activity of the samples was contained in the polar fraction (eluted with acetone), and the EEq was calculated from those polar eluates. Likewise with the E-Screen, almost all estrogenic activity (94 – 100%) of the sample was eluted with acetone.

### 4.4.2. Estrogenicity profile along the treatment train

Estrogenicity of the samples (expressed as EEq) during the treatment train is shown in Fig.

4.2. The two bioassays yielded somewhat different results, although both showed a significant decrease of EEq during treatment and could not detect estrogenic chemicals in the final treated effluent (Fig. 4.2).

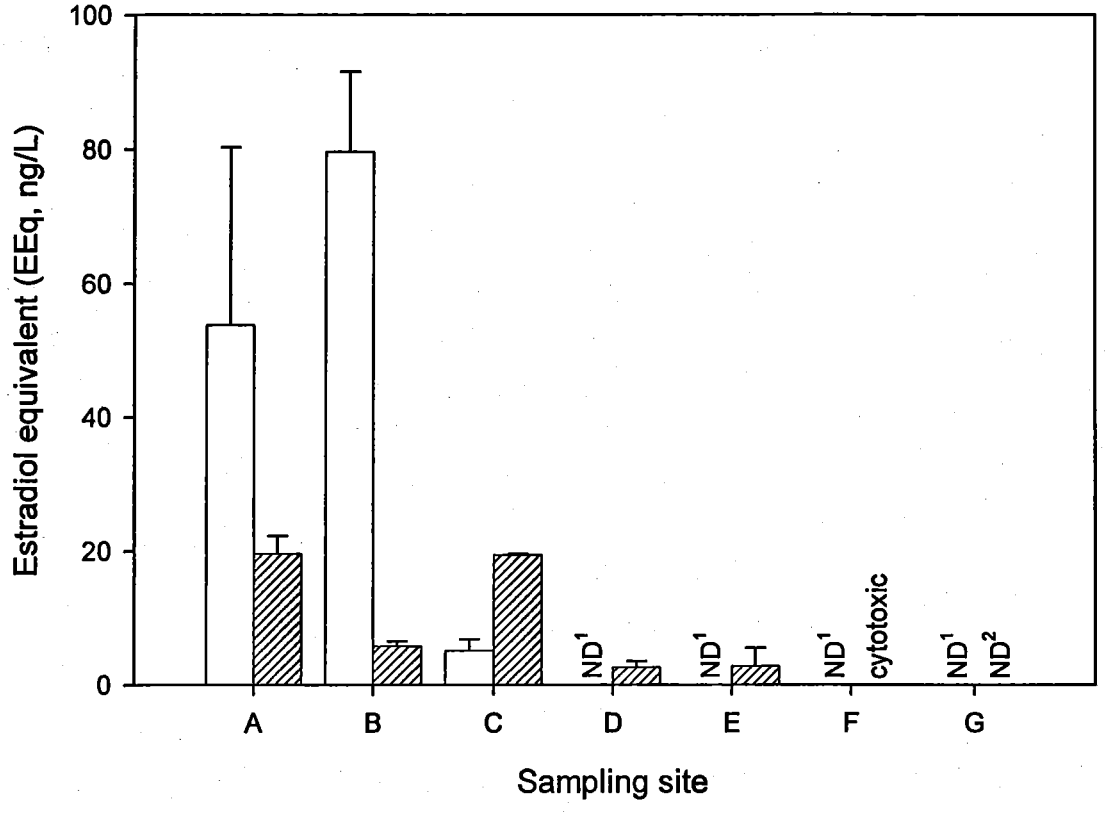


**Figure 4.2:** Estradiol equivalents (EEq ± SE) of the samples determined by ER binding assay (□, n=3) and E-Screen (▨, n=2) at each of the sampling points defined in Fig. 4.1. Method detection limit for the ER binding assay ($ND^1$) was 0.75 ng/L. Method detection limit for the E-Screen ($ND^2$) was 0.03 ng/L.

With the ER binding assay (Fig. 4.2, open bars), EEq in the influent was 54 ± 27 ng/L. This level increased to 80 ± 12 ng/L after primary settling and anaerobic selection (Fig. 4.2, sample B), but decreased to 5.1 ± 1.7 ng/L after aerobic sludge treatment and nitrification/denitrification (Fig. 4.2, sample C). After secondary settling, the level dropped below detection limit (< 0.75 ng/L) (Fig. 4.2, samples D to G).

With the E-Screen (Fig 4.2, hatched bars), EEq in the influent was 20 ± 2.7 ng/L (Fig. 4.2, sample A) of a fully agonistic $E_2$ mimic (RPE > 80%, Table 4.1, sample A). Following

primary settling and anaerobic selection, EEq decreased by 72% to 5.8 ± 0.8 ng/L (Fig. 4.2, sample B), but later increased to 19 ± 0.2 ng/L after aerobic sludge treatment and nitrification/denitrification (Fig. 4.2, sample C). The estrogenic chemicals were however only weakly agonistic (RPE < 50%, Table 4.1, samples C to E). After secondary settling, EEq level dropped markedly to 2.6 ± 0.9 ng/L (Fig. 4.2, sample D) and remained unchanged following sand filtration (Fig. 4.2, sample E). The effluent from the ozone contact tanks was cytotoxic to MCF-7 cells up to a dilution volume of 0.5 L, revealing cell counts significantly lower than the hormone-free negative control (Fig. 4.2, sample F), and thus EEq could not be determined for that sample. The level of EEq in UV-treated effluent was below the limit of detection of 0.03 ng/L (Fig. 4.2, sample G).

**Table 4.1:** Estrogenic activity relative to 17β-estradiol (relative proliferative effect, RPE) of the samples in the E-Screen. Each value represents mean ± SD of two independent assays.

| Sample | RPE (%) | Interpretation |
|---|---|---|
| A | 86 ± 14 | Full agonism |
| B | 73 ± 10 | Partial agonism |
| C | 35 ± 9.3 | Partial agonism |
| D | 28 ± 0.3 | Partial agonism |
| E | 46 ± 5.8 | Partial agonism |
| F | N.A. | N.A. |
| G | N.A. | N.A. |

N.A. = not active

The relative potencies of $E_2$, $E_1$, and $EE_2$ relative to $E_2$ in the sheep ER binding assay and the E-screen (as reported in Körner et al. 1999) are presented in Table 4.2. $EE_2$ was not detectable by GC/MS in any of the three selected samples for chemical analysis (data not shown, limit of reporting of 5 ng/L). $E_2$ and $E_1$ in the influent were 19 and 45 ng/L, respectively (Table 4.3, sample A), and below the limit of reporting (LOR) after secondary settling (< 5 ng/L, Table 4.3, samples D to G).

**Table 4.2:** Octanol-water partition coefficient ($K_{OW}$) and relative potency of selected estrogens and estrogen mimics compared to 17β-estradiol in the sheep ERBA and the E-Screen.

| Compound | CAS RN | log $K_{OW}$ | ER binding assay RBA[2] | E-Screen RP[3] |
|---|---|---|---|---|
| 17β-Estradiol ($E_2$) | 50-28-2 | 3.94[1] | 1.0 | 1.0[4] |
| Estrone ($E_1$) | 53-16-7 | 3.43[1] | 0.15 | 0.096[4] |
| Ethynylestradiol ($EE_2$) | 57-63-6 | 4.15[1] | 1.9 | 0.91[4] |
| *p*-Nonylphenol | 84852-15-3 | 4.48[2] | 0.000 074 | 0.000 076[4] |
| 4-*t*-Octylphenol | 140-66-9 | 4.12[2] | 0.000 34 | 0.000 076[4] |
| Bisphenol A | 80-05-7 | 3.32[3] | 0.002 3 | 0.000 053[4] |

CAS RN = Chemical abstracts service registry number. RBA = relative binding affinity, RP = relative potency.

Data from [1] Lai et al. (2000), [2] Ahel and Giger (1993), [3] Ying et al. (2003), [4] Körner et al. (1999).

**Table 4.3:** Concentration of 17β-estradiol ($E_2$) and estrone ($E_1$) in selected samples determined by GC/MS and predicted EEq evaluated from the ER binding assay and E-Screen.

| Sample | $E_2$ (ng/L) | $E_1$ (ng/L) | Predicted ERBA EEq (ng/L) [% actual] | Predicted E-Screen EEq (ng/L) [% actual] |
|---|---|---|---|---|
| A | 19 | 45 | 25.8 [31-95%] | 23.3 [102-134%] |
| D | < LOR | < LOR | – | – |
| G | < LOR | < LOR | – | – |

LOR = limit of reporting, 5 ng/L.

## 4.5. Discussion

### 4.5.1. Raw sewage

The concentrations of estrogenic compounds present in raw sewage at the Landsborough STP were comparable to those reported by previous studies for municipal sewage treatment plants abroad (Desbrow et al. 1998, Shore and Shemesh 2003). As in previous studies, most of the activity was associated with the most polar fraction, where compounds like $E_2$, $E_1$, and $EE_2$ are found (Desbrow et al. 1998, Snyder et al. 2001). In municipal sewage, these compounds originate from human excretion and are therefore present wherever humans are. With the E-Screen, the predicted EEq from the natural hormones $E_2$ and $E_1$ alone was 102 - 134% of the actual EEq of raw sewage (Table 4.3). With the ERBA, they accounted for 31 - 95% of the activity (Table 4.3; the wide spread is due to the large amount of variability in estrogenicity of sample A in the ERBA). The difference between the two assays suggests the presence of other chemicals that bind to the ER but are not potent inducers of estrogenic effects (such as nonylphenol, bisphenol A, and octylphenol; Table 4.2) which could account for the remainder of the activity measured in the bioassay.

At the highest concentration tested, chemicals in the raw sewage interfered with the ER binding assay. It is postulated that the high lipid content in raw sewage may have artificially increased non-specific binding at the highest concentration, an effect that disappeared after dilution of the sample. This interference with the assay resulted in more variability for the EEq levels in the influent than for other samples (Fig. 4.2, sample A).

### 4.5.2. Primary and secondary treatment

The slight increase in EEq levels measured with the ER binding assay and the concomitant decrease in EEq in the E-Screen after primary treatment and anaerobic sludge treatment (Fig. 4.2, sample B) suggest either the formation of a less potent chemical with a higher affinity for the ER or the presence of large quantities of a much less potent chemical with less affinity for the ER. A combination of reactivation of steroid estrogens by cleavage of the glucuronide conjugates during primary treatment (Ternes et al. 1999a) with the degradation of $E_2$ into $E_1$ early in activated sludge treatment (Ternes et al. 1999b) could be one of the explanations for this phenomenon.

Aerobic sludge treatment and post nitrification/denitrification decreased EEq levels measured by the ER binding assay by 96% (Fig 4.2, open bars, sample C). At the same time, EEq levels in the E-Screen increased appreciably, but only with partial agonistic effects (RPE=35%; Table 4.1). The combination of low binding to the ER with low RPE suggests the presence of weakly estrogenic substances (*e.g.*, phenols and derivatives, phthalates, pesticides) and/or of estrogen antagonists.

The combination of primary and secondary treatments removed 87 and 98% of the initial estrogenic activity from the aqueous phase (E-Screen and ER binding assay, respectively; Fig. 4.2, sample D). These figures are similar to those reported for municipal STPs in other parts of the world (Shore and Shemesh 2003), and clearly indicate that activated sludge treatment is very effective at removing estrogenic activity from sewage water. Based on GCMS results, at least 74% of all $E_2$ and 89% of all $E_1$ was removed by secondary treatment (Table 4.3). However, quantification of the removal efficacy for individual compounds with GC/MS analysis was not very useful because of the relatively high limit of reporting of this method (5 ng/L).

In a study on the fate of estrogens during sewage treatment, Andersen *et al.* (2003a) showed that most estrogens were either eliminated or bound to sludge during activated sludge treatment. Estrogen concentrations in the sludge were not measured in this study, but based on their relatively high $K_{OW}$ values (Table 4.2) a similar scenario would be expected. Further studies at this plant will investigate estrogen concentrations in the sludge.

### 4.5.3. Tertiary treatment

The cytotoxicity of the effluent extract after ozonation to MCF-7 breast cancer cells (sample F) could be the result of the formation of some toxic ozonation by-products. The sewage samples before and after sand filtration (samples D and E) were very similar in their estrogenicity, indicating this treatment step may not be efficient in the removal of estrogenic substances. A comparable result with slow sand filtration as tertiary treatment step was found in a municipal sewage treatment plant in Bavaria, southern Germany (Körner, unpublished results).

### 4.5.4. Overall efficacy of the treatment plant

Full treatment removed in excess of 95 to 98% of all estrogenic activity, depending on the bioassay (Fig. 4.2, E-Screen and ER binding assay, respectively), and estrogenicity was below detection limit of the bioassays in the final effluent (Fig. 4.2 and Table 4.3, sample G) clearly indicating that treatments successfully removed and/or sequestered most of those compounds.

In laboratory exposures, median effective concentrations for a significant induction of the egg-yolk precursor vitellogenin in juvenile rainbow trout after 2 weeks of exposure were estimated to be 10 - 20 ng/L for $E_2$, 25 - 60 ng/L for $E_1$, and 1 ng/L for $EE_2$ (Routledge et al. 1998, Thorpe et al. 2003). Although the analytical detection limit for $EE_2$ was higher than this level (5 ng/L), the fact that the overall estrogenic potency of the effluent samples was below detection limit of the bioassays (0.75 ng/L in the ERBA, 0.03 ng/L in the E-Screen) suggests that the potential for the effluent to induce estrogenic effects in exposed wildlife is very small, at least in the short term. Further studies need to be undertaken to examine the long-term effects of treated sewage containing trace concentrations (ppt) of estrogenic chemicals on exposed wildlife, as well as to determine if the unique Australian wildlife is more susceptible to estrogenic chemicals.

The samples in this study were taken on one day, and further sampling is required to provide a more complete understanding of the day-to-day variation in estrogenicity at that plant. It is also important to note that the high level of tertiary treatment in place at the Landsborough STP is not representative of municipal STPs in Australia, which are often limited to secondary treatment followed by disinfection. A survey of estrogenic compounds in sewage at several STPs in Queensland and New Zealand will be published shortly.

## 4.6. Co-author contributions

This chapter in press in Environmental Science and Technology. Besides supervisory committee members, the co-author for this paper is Wolfgang Körner (Institut für Organische Chemie, Universität Tübingen, Germany). Wolfgang carried out all E-Screen assays. GC/MS analyses were carried out by the Australian Government Analytical Laboratory (Pymble, NSW, Australia).

# 5. Bioassay-derived androgen and estrogen concentrations of municipal sewage in Australia and New Zealand

## 5.1. Abstract

Raw sewage and sewage at various stages of treatment was sampled from 15 municipal sewage treatment plants in south Queensland (Australia) and Canterbury (New Zealand). Estrogenic and androgenic activity was determined with a sheep estrogen receptor and a rainbow trout androgen receptor binding assays, respectively. The raw sewage influents contained significant levels of both estrogenic (<4 – 185 ng/L estradiol equivalents) and androgenic activity (1920 – 9330 ng/L testosterone equivalents). Subsequent treatment of raw sewage successfully removed most of the activity so that the estrogenicity and androgenicity associated with the final effluents were very low (<1 – 4.2 ng/L estradiol equivalents and <6.5 – 736 ng/L testosterone equivalents, respectively). Secondary treatment was the most effective treatment step to remove estrogenic and androgenic activity from sewage water. Activated sludge treatment in particular removed 92% to >99% of the estrogenic activity and 82% to >99% of the androgenic activity in sewage.

## 5.2. Introduction

Sexual development in vertebrates is coordinated by hormones, and steroid hormones such as estrogens and androgens play a critical role in sex differentiation and sexual development (Hadley 1988). Several studies in the United Kingdom have shown that wild fish exposed to treated sewage water exhibit reproductive abnormalities consistent with exposure to estrogens or estrogen-mimics (Purdom et al. 1994, Harries et al. 1996, Jobling et al. 1998, Jobling and Tyler 2003). A toxicity identification and evaluation (TIE) revealed that natural and synthetic hormones excreted by humans as well as some alkylphenolic industrial chemicals present in treated sewage were responsible for the majority of the estrogenic activity (Desbrow et al. 1998, Routledge et al. 1998). This problem is not confined to United Kingdom rivers, and studies in continental Europe, Japan, and North America have confirmed that treated sewage there also contains chemicals with estrogenic activity (Solé et al. 2000, Körner et al. 2001, Onda et al. 2002) and that these may be impacting a wide range of wild fish species (Folmar

et al. 1996, Hashimoto et al. 2000, Folmar et al. 2001b, Christiansen et al. 2002, Jobling and Tyler 2003). Androgenic hormones have recently been found in treated sewage (Kirk et al. 2002) and river water (Thomas et al. 2002), and this has raised concerns about possible androgenic effects of treated sewage.

There have been few surveys on the concentrations of estrogens or androgens in sewage in Australia or New Zealand. The anthropogenic source of these chemicals means that differences in population densities, treatment technology, and socio-economic factors may have a significant influence on their levels in the environment. Climatic differences can also complicate extrapolation of data from one region to the next. For example, Layton et al. (2000) showed that temperature can significantly affect the rate of degradation of hormones during activated sludge treatment. Freshwater supply is fast becoming a major environmental issue in Australia and on the Canterbury Plains of New Zealand. A thorough assessment of the efficacy of sewage treatment plants (STPs) to remove bioactive compounds is required as a first step towards understanding the environmental risk of wastewater utilisation.

The primary aim of this research was to measure estrogenic and androgenic activity of raw and treated sewage from several municipal STPs. Estrogen and androgen concentrations were measured with an estrogen and an androgen receptor binding assay. The efficacy of treatment was also compared between the different STPs to identify which treatment steps were most effective at reducing the estrogenic and androgenic activity in sewage.

## 5.3. Materials and Methods.

### 5.3.1. Sampling

Thirteen STPs in south Queensland (Australia) and two STPs in Canterbury (New Zealand) were sampled (see Table 5.1 for type of STP, sampling dates, and environmental conditions at time of sampling). Sewage samples were collected in 1-L methanol-rinsed glass Schott bottles and brought back to the laboratory on ice, where they were kept at 4°C and extracted using reversed solid-phase extraction (SPE) cartridges within 24h.

### 5.3.2. Extraction

All glassware was methanol-rinsed. Oasis HLB cartridges (Waters Corp., Milford, MA, USA) were preconditioned with 2×3 mL methanol, followed by equilibration with 2×3 mL water. Prior to extraction, sewage samples were centrifuged at 4500×*g* for 30 min at 4°C to remove large particulate matter; 900 mL of the supernatant was decanted into a glass measuring cylinder, and transferred to a glass flask. The samples were then passed through preconditioned SPE cartridges dropwise by vacuum suction (maximum of 70 kPa). The cartridges were then dried on the manifold for 10 min, capped, wrapped in aluminium foil, and stored at -20°C until elution. The cartridges were eluted with 2×3 mL methanol. The eluate was evaporated at 50°C under gentle nitrogen stream, and reconstituted in 250 µL of methanol. The reconstituted samples were kept in small amber glass vials at -20°C until analysed in the bioassays.

### 5.3.3. Bioassays

Two *in vitro* competitive receptor binding assays were used to determine the total estrogenic and androgenic activity of the extracted sewage samples. Estrogen receptors were isolated from sheep uteri based on the protocol described in Tremblay et al. (2004). Briefly, sheep uteri were excised from sheep at the abattoir and brought back to the laboratory in ice-cold isotonic saline solution (0.9% NaCl). The tissue was trimmed of fat, snap-frozen in liquid nitrogen, and stored for up to 1 month at -80°C until further processing. All manipulations were carried out in a cold room at 4°C to minimize denaturing the receptor proteins. The uterine tissue was thawed in TEDG buffer (10 mM tris base, 2 mM EDTA, 10% glycerol, 1 mM dithiothreitol, pH 7.2 at 4°C), minced with a razor blade, and homogenized (polytron) in TEDG buffer (50 mg tissue/mL) for three strokes of 3 s at 10 000 rpm. The homogenate was centrifuged at 1000×*g* for 10 min at 4°C, and the supernatant transferred to an ultracentrifuge tube and centrifuged at 105,000×*g* for 50 min at 4°C. The final supernatant, containing cytosolic estrogen receptors (ER), was aliquoted and stored at -80°C until use. This method typically yields an ER preparation with saturable binding capacity ($B_{max}$ = 570 ± 150 fmol/mg of protein) with high affinity to estradiol ($E_2$) ($K_d$ = 0.17 ± 0.01 nM).

Androgen receptors (AR) were isolated from rainbow trout brains based on the protocol described in Sperry and Thomas (1999a) and Bandelij (2003). Briefly, W buffer (50 mM

Tris-HCl, 1.5 mM $Na_2EDTA$, 1 mM dithiothreitol, 30% glycerol, pH 7.5 at 4°C), H buffer (W buffer with 0.5 mM Pefabloc SC), and E buffer (H buffer with 0.7 M KCl) were prepared fresh. Adult male rainbow trouts were anaesthetized with tricaine methanesulfonate (MS222, 0.1 g/L) and killed by spinal severance. Brains were excised and homogenized in two volumes of ice-cold H buffer with three passes of a Potter-Elvehjem teflon-pestle homogenizer. The homogenates were pooled and centrifuged at 2500×*g* for 15 min at 4°C. The pellet was washed twice with W buffer, and extracted in E buffer at 500 mg/mL for 1h with occasional stirring. The extract was then centrifuged at 100,000×*g* for 60 min at 4°C, and the final supernatant containing the nuclear AR was aliquoted and stored at -80°C until use. This method typically yields an AR preparation with saturable binding capacity ($B_{max}$ = 1.1 pmol/mg of protein) with high affinity to testosterone (T) ($K_d$ = 0.41 nM).

The competitive receptor binding assays were adapted from Shelby et al. (1996) and ICCVAM (2003). In brief, a serial dilution of the sewage sample extract (100 µL in the ER, 50 µL in the AR assay) was incubated in glass tubes with a known concentration of radiolabeled native ligand for the receptor (100 µL for 0.5 nM [2,4,6,7-$^3$H]$E_2$ in the ER, 50 µL for 2.5 nM [1,2,6,7-$^3$H]T in the AR assay), a standard number of receptors (50 µL in the ER, 15 µL in the AR assay), and TEDG buffer (150 µL in the ER, 135 µL in the AR assay), for a final volume of 400 µL in the ER and 250 µL in the AR assay. After 18h at 4°C, dextran-coated charcoal (DCC; 0.5% w/v charcoal, 0.05% w/v dextan T70, in TEDG buffer) was added to each tube (200 µL in the ER, 500 µL in the AR assay) to strip any unbound radioligand from the supernatant. After a brief incubation at 4°C (12 min in the ER, 5 min in the AR assay), the tubes were centrifuged (1500×*g* for 12 min in the ER, 2000×*g* for 15 min in the AR assay) at 4°C, and the supernatant (450 µL in the ER, 550 µL in the AR) was pipetted into a scintillation counting tube. Liquid scintillation cocktail was added (2.5 mL Ultima Gold [Packard] in the ER, 5 mL of scintillation cocktail [2 L toluene, 1 L Triton X-100, 12 g 2,5,-diphenyloxazole (PPO), 0.6 g 1,4-bis[2-5-phenyloxazolyl]benzene (POPOP)] in the AR assay), and β-radiation measured by liquid scintillation (in a Wallac 1490 for the ER, and Packard TriCarb 2100-TR for the AR assay).

A displacement curve was obtained by plotting the proportion of radioligand still bound to the receptor against the equivalent volume of sewage tested. The equivalent volume required to displace 50% of the radioligand from the receptor binding sites, or $EV_{50}$, was calculated by fitting a Verhulst curve to the data by least-square regression using an Excel9 module written

by F. Leusch, where bottom is the minimum and top is the maximum $x$ value:

$$\text{Verhulst equation: } y = \frac{bottom + (top - bottom)}{1 + 10\text{^}[(\log EV_{50} - \log x) \times slope]}$$

The activity (estrogenic or androgenic) of the sample was evaluated by comparing the $EV_{50}$ of the sample with the $EC_{50}$ (effective concentration for 50% displacement of the native radioligand from the receptor site) of a standard curve (with $E_2$ in the ER, and T in the AR assay) that was run in every assay. The potency of the sewage samples was expressed as estradiol or testosterone equivalents (EEq and TEq, respectively), the ratio between the amount of the native ligand in the incubation tube at $EC_{50}$ (in ng) and the $EV_{50}$ (in L). With 1 L of sewage sample, the method detection limit was 1 ng/L for the ER and 6.5 ng/L for the AR assay.

## 5.4. Results

Estrogenic and androgenic activity of all sewage samples is summarized in Table 5.1. Raw sewage was highly estrogenic in the ER binding assay, with EEq between <4 and 185 ng/L. There was a slight increase in estrogenic activity in four out of six STP samples after primary treatment and a sharp decrease in the Eeq concentration in all STP samples after secondary treatment (Table 5.1). EEq in the final effluent varied from <1 to 4.2 ng/L. A constructed wetland for tertiary treatment of effluent from plant F1A also reduced the estrogenic activity from 6.4 to <1 ng/L (Table 5.1, F1A).

Raw sewage was also highly androgenic in the AR assay, with TEq between 1920 and 9330 ng/L. TEq in secondary-treated sewage at plant F1A was still relatively high (2290 ng/L). TEq in the final effluent of the other plants tested was from <6.5 to 736 ng/L (Table 5.1). The overall removal efficacy for both estrogenic and androgenic activity at all plants is shown in Fig. 5.1.

**Table 5.1:** Estradiol equivalents (EEq) and testosterone equivalents (TEq) for all 15 sewage treatment plants tested determined with sheep uterine estrogen and rainbow trout brain androgen receptor binding assays, respectively. Raw = raw sewage; 1°Treat = primary-treated sewage; 2°Treat = secondary-treated sewage; Plt Eff = plant effluent; L/HP/W = lagoon, holding pond, or wetland; Fin Eff = final effluent.

| STP | STP Type[a] | Flow ($m^3$/d) | Head (people) | Source[b] | Sample date | Air temp.[c] (°C) | Rain[c] (mm) | EEq[d] (ng/L ± SE) | | | | | | TEq[e] (ng/L) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Raw | 1°Treat | 2°Treat | Plt Eff | L/HP /W | Fin Eff | Raw | Plt Eff | Fin Eff |
| S1A[f] | S | 330 | 1250[g] | D | 7/03 | 15 – 23 | 2.2 | 50 ± 20 | NT | NT | BDL | BDL | NT | 3360 | 196 | NT |
| S1A[f] | S | 194 | 750[g] | D | 7/03 | 14 – 23 | 1.5 | 29 ± 12 | NT | NT | BDL | BDL | NT | 4890 | 208 | NT |
| S2A[f] | S | 113 | 430[g] | D | 7/03 | 15 – 23 | 2.2 | < 4 | NT | NT | BDL | NT | NT | NT | NT | NT |
| S2A[f] | S | 109 | 420[g] | D | 7/03 | 14 – 23 | 1.5 | 15 ± 6.8 | NT | NT | BDL | NT | NT | 2570 | NT | BDL |
| S3A | S | 980 | 3 800[g] | D | 8/00 | 9 – 25 | 0.0 | 54 ± 26 | 80 ± 12 | BDL | BDL | NT | NT | NT | NT | NT |
| S3A | S | 980 | 3 800[g] | D | 8/02 | 8 – 22 | 0.0 | 76 ± 11 | NT | NT | NT | NT | NT | NT | NT | NT |
| S3A | S | 980 | 3 800[g] | D | 7/03 | 9 – 23 | 0.0 | 93 ± 5.0 | NT | NT | < 4 | NT | NT | 1920 | NT | 105 |
| S4A | S | 1 500 | 6 000 | D | 7/03 | 8 – 20 | 0.2 | 125 ± 8.5 | NT | < 4 | BDL | NT | NT | 6810 | NT | 603 |
| S5A | S | 5 900 | 23 000[g] | D | 7/03 | 14 – 22 | 4.2 | 137 ± 16 | 61 ± 13 | 6.0 ± 4.1 | BDL | NT | NT | NT | NT | NT |
| S6A | S | 3 500 | 13 000[g] | D | 7/03 | 14 – 22 | 4.2 | 163 ± 1.5 | NT | < 4 | 4.2 ± 1.7 | NT | NT | 6920 | NT | BDL |
| S7A | S | 58 000 | 220 000 | D, I | 8/02 | 7 – 24 | 0.0 | 62 ± 15 | NT | NT | NT | NT | NT | NT | NT | NT |
| S7A | S | 58 000 | 220 000 | D, I | 7/03 | 9 – 22 | 0.0 | 64 ± 9.3 | 75 ± 17 | < 4 | NT | NT | NT | 9330 | NT | NT |
| S8A | S | 6 800 | 26 000[g] | D, i, b | 7/03 | 11 – 21 | 0.2 | 81 ± 30 | 89 ± 29 | < 4 | < 4 | NT | NT | 2630 | NT | 94.9 |
| S9A | S | 130 000 | 750 000 | D, I | 7/03 | 13 – 21 | 0.0 | 51 ± 6.9 | 64 ± 19 | NT | BDL | NT | NT | 4630 | NT | 84.5 |
| S10A | S | 12 000 | 45 000 | D, I | 7/03 | 11 – 21 | 0.2 | 66 ± 18[h] | NT | NT | BDL | NT | NT | 4150 | NT | 736 |
| S11A | S | 26 000 | 100 000[g] | D | 7/03 | 5 – 23 | 2.8 | NT | 77 ± 3.4 | NT | < 4 | NT | NT | NT | NT | NT |
| S12A | S | 60 000 | 230 000[g] | D, i | 7/03 | 6 – 22 | 4.2 | NT | NT | NT | < 4 | NT | NT | 2460 | NT | 214 |
| F1A | F | 570 | 2 200 | D | 6/03 | 14 – 21 | 1.6 | 83 ± 30 | NT | NT | 6.4 ± 3.0 | < 4 | BDL | 5340 | 2290 | NT |
| F2N | F | 161 000 | 320 000 | D, i | 9/03 | 4 – 14 | 2.0 | 127 ± 14 | 55 ± 23 | 143 ± 15[h] | NT | BDL | NT | NT | NT | NT |
| O1N | O | 4 000 | 10 500 | D | 9/03 | 4 – 14 | 2.0 | 185 ± 31 | NT | NT | NT | BDL | BDL | NT | NT | NT |

[a] Type of secondary treatment: (F) fixed film (trickling filter); (S) suspended growth (activated sludge); and (O) oxidation ponds.

[b] Source of influent are domestic (D), industrial (I), and biomedical (B). A lowercase letter indicates this type of wastewater contributes less than 15% of the total influent flow.

[c] Minimum and maximum temperature and rainfall at the closest monitoring station over a 48h period prior to sampling. Australian data courtesy of the Commonwealth Bureau of Meteorology; New Zealand data courtesy of the National Institute of Water and Atmospheric Research.

[d] Values are the mean of assays with receptors isolated from 2 different sheep (each done in duplicate) ± SE. Accurate quantification limit was 4 ng/L, and method detection limit was 1 ng/L. Samples below detection limit are indicated as "BDL", samples where activity was detected but was too low to be accurately quantified are marked as "< 4".

[e] Value is the mean of duplicates in an assay with pooled receptors from several male rainbow trout. Method detection limit was 6.5 ng/L. Samples below detection limit are indicated as "BDL".

[f] Indicates this plant is located in a tourist area, and flow can vary depending on tourism season.

[g] Actual head not available, equivalent population (EP) estimated based on average daily water use per person in Australia (260L/person/d).

[h] 24 h-composite sample.

NT = Not tested

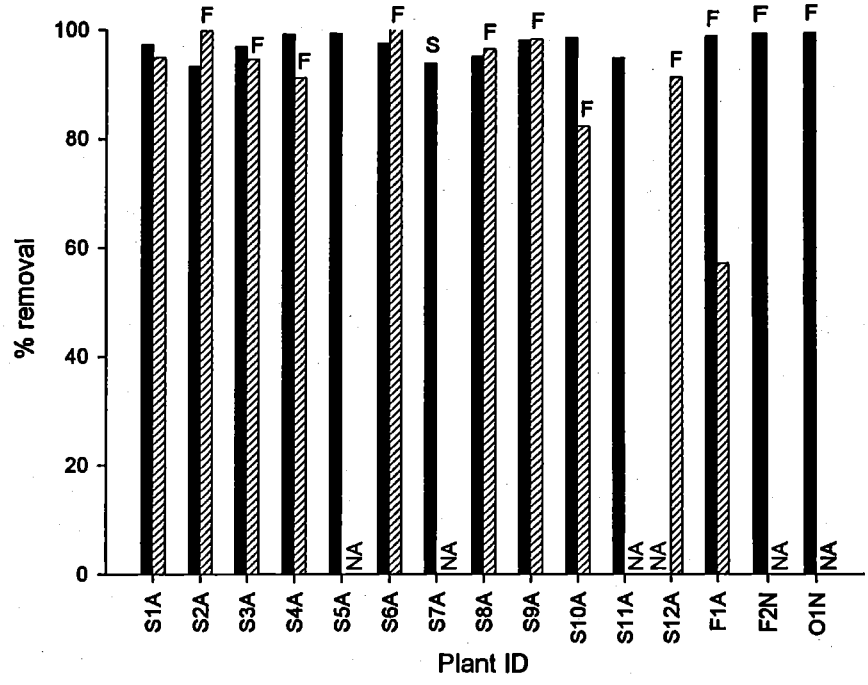


**Figure 5.1:** Removal efficacy for estrogenicity (full bar) and androgenicity (hatched bar) at each of the 15 tested sewage treatment plants, expressed as percent removal from raw sewage to plant effluent, except when marked with S where it is until secondary treatment, and F where it is until final effluent (Table 5.1). Plant ID refers to plant identification in Table 5.1. NA = not available.

## 5.5.Discussion

### 5.5.1. Estrogenicity of sewage

All raw sewage samples tested in this study displayed significant estrogenic activity, from <4 to 185 ng/L EEq (Table 5.1, Raw). These are very similar to levels reported in other studies. For example, levels of 1 – 120 ng/L EEq were reported in raw sewage in the Netherlands (ER-CALUX assay, Murk et al. 2002), 35 – 72 ng/L in Japan (yeast assay, Onda et al. 2002), 58 – 70 ng/L in Germany (E-Screen, Körner et al 2000), and 20 – 80 ng/L in the United Kingdom (yeast assay, Kirk et al. 2002). The sewage samples in this study were from municipal treatment plants that service highly urbanized areas, and natural hormones are most likely the source of the estrogenic activity. Preliminary chemical analyses with these samples suggest that 17β-estradiol, estrone, and estriol are responsible for more than 80% of the total estrogenic activity (data not shown). Although estrogens such as $E_2$ are excreted from humans in an inactive form either as glucuronide or sulfonite conjugates (Tietz 1987), microbial activity in the sewerage system and during the early steps of treatment results could deconjugate these compounds and reactivate the steroid hormones (Ternes et al. 1999a, Baronti et al. 2000). This study supports this hypothesis because estrogenic activity was often increased after primary treatment (Table 5.1).

Secondary treatment was very effective at reducing the estrogenic activity in sewage. With the exception of plant F2N, secondary treatment removed more than 90% of the activity in primary-treated sewage, in most cases to levels below quantification limit (<4 ng/L EEq). Activated sludge treatment was particularly effective and removed 92% to >99% of the estrogenic activity in the raw sewage (Table 5.1). Activated sludge has previously been shown to be very effective at removing estrogen hormones and other lipophilic contaminants from the water phase (Baronti et al. 2000, Byrns 2001, Joss et al. 2004). Removal of estrogenic activity from the water phase is a combination of degradation and sorption to sludge particles (Layton et al. 2000, Andersen et al. 2003a). This is supported by the findings of high levels of estrogenic activity in sludge at municipal STPs in the Netherlands (Murk et al. 2002). Sludge was not sampled in this study, but future studies will investigate whether the reduced estrogenic activity in effluent represents an overall loss of activity or merely the transfer of activity to the sludge component.

The removal efficacy of secondary treatment at plants with trickling filters was much more variable. The Australian trickling filter STP (F1A) removed 92% of the estrogenic activity,

while the New Zealand trickling filter STP (F2N) actually caused an increase in estrogenic activity (Table 5.1), most likely due to late reactivation of the conjugated steroids. These results are similar to those reported in Giger et al. (1984), where trickling filters were found to be less efficient than activated sludge systems at removing 4-nonylphenol (a lipophilic contaminant with estrogenic activity). This is most likely due to lower sorption of bioactive compounds to the sludge in fixed-film systems (such as trickling filters). The poor efficacy of the New Zealand trickling filter (F2N) compared with the Australian plant (F1A) may be related to the lower ambient air temperature (4 – 14°C at the New Zealand plant compared with 14 – 21°C at the Australian plant; Table 5.1). For example, Mann and Reid (1971) showed that degradation of lipophilic contaminants in trickling filters decreased from 80% at 15°C to 20% at 5°C.

In most cases, the plant effluent was discharged into a holding pond or wetland where further degradation of the estrogenic chemicals took place (Table 5.1) before discharge of the effluent into the environment. Natural ($E_2$ and estrone) and synthetic hormones (ethinylestradiol) that are responsible for most of the estrogenic activity in domestic sewage (Desbrow et al. 1998, Routledge et al. 1998) are also sensitive to photodegradation (Jürgens et al. 2002). Shallow oxidation ponds (present at plants F2N and O1N) and the wetland at plant F1A were very effective at removing the remaining estrogenic activity in municipal sewage to levels below detection limit (< 1 ng/L EEq) (Table 5.1).

Overall, sewage treatment was very effective at removing the estrogenic activity (Fig. 5.1). Estrogenicity in the final effluent was below detection limit (< 1 ng/L) at nine of the 15 STPs tested, below quantification limit (< 4 ng/L) at five of the STPs, and at 4.2 ng/L EEq at one of the STPs tested (S6A, Table 5.1). These levels are relatively low compared with other studies, where estrogenic activity in final effluents of <1 – 16 ng/L in the Netherlands (ER-CALUX assay, Murk et al. 2002), 4 – 35 ng/L in Japan (yeast assay, Onda et al. 2002), 6 ng/L in Germany (E-Screen, Körner et al. 2000), and <3 – 13 ng/L in the United Kingdom (yeast assay, Kirk et al 2002) have been reported.

In laboratory exposure studies, the median effective concentration of $E_2$ required for a significant induction of the egg-yolk precursor vitellogenin in juvenile rainbow trout after 2 weeks of exposure was estimated to be 10 – 20 ng/L (Routledge et al. 1998, Thorpe et al. 2003). Exposure to 10 – 100 ng/L of $E_2$ for 110 d induced intersex in adult male Japanese medaka (*Oryzias latipes*) (Metcalfe et al. 2001). The levels of estrogenic activity in undiluted

final effluents (expressed in estradiol equivalents, EEq) were below 4.2 ng/L for all plants tested in this study. After the dilution effect associated with discharge into the environment, the potential for estrogenic effects in exposed wildlife was minimal.

### 5.5.2. Androgenicity of sewage

Androgenic activity in raw and treated sewage was on average 50 – 100-fold higher than estrogenic activity (Table 5.1). As suggested in Kirk et al. (2002), most of the androgenic activity in municipal sewage with a predominantly domestic input is most likely caused by androgens excreted by humans. Androgen levels in humans are generally much higher than estrogen levels. For example, plasma testosterone (T) levels are 3000 – 10,000 ng/L in adult males and 600 – 3000 ng/L in adult females, while $E_2$ plasma concentrations are usually 10 – 60 ng/L in adult males and 30 – 400 ng/L in adult females (Tietz 1987). Concentrations of androgens in sewage would therefore be expected to be much higher than those of estrogens. Androgenic activity in raw sewage was in the same range as that reported for several STPs in the United Kingdom, which ranged from 113 to 4300 ng/L (DHT equivalents determined by yeast assay, Kirk et al. 2002). As was the case with the estrogenic activity, STPs with activated sludge treatment were more effective than trickling filters at removing the androgenic activity, with 82% to >99% net removal in activated sludge plants compared to 57% in the trickling filter plant (F1A) (Table 5.1). Similar results were reported for STPs in Sweden (Svenson and Allard 2004). In batch experiments using spiked testosterone and sludge from four municipal STPs, 55 – 65% of testosterone added was mineralized within 90 min (Layton et al. 2000). Similar to estrogens, sorption to activated sludge appears to be the major mechanism in removing androgens from the aqueous phase (Layton et al. 2000, Esperanza et al. 2004). The androgenic activity in secondary-treated sewage at plant F1A was very high (Table 5.1). However, this plant effluent is first discharged into a constructed wetland. Passage through the wetland, which takes approximately 14 d, was very efficient at removing the estrogenic activity of the plant effluent before final discharge (Table 5.1). Previous studies at that same wetland have also shown it to be very effective at removing a wide range of endocrine-disrupting chemicals (Chapman 2003), and the activity in the final effluent is expected to be within the range reported for the other plants (Table 5.1, Fin. Eff.). The androgenic activity in the final effluents was still relatively high (<6.5 – 736 ng/L TEq), at levels similar to those reported for STPs in other studies. In the United Kingdom for

example, levels of <113 – 4000 ng/L (DHT equivalents in a yeast assay, Kirk et al. 2002) and 34 – 635 ng/L (DHT equivalents in a yeast assay, Thomas et al. 2002) were reported in final effluents of several STPs. Little is known about the effect of exposure of fish to androgenic chemicals. The lowest observable effective concentration for induction of the male-specific protein spiggin in female sticklebacks (*Gasterosteus aculeatus*) after 3 – 5 weeks of exposure to dihydrotesterone was 2000 – 3000 ng/L (Katsiadaki et al. 2002), suggesting that fish may not be susceptible to androgenic chemicals below the µg/L level. However, some studies have shown masculinization of mosquitofish exposed to paper mill effluents containing ng/L levels of the steroid androstenedione (Jenkins et al. 2001, Ellis et al. 2003), and more research is needed to determine if the androgenic activity reported in the present study is sufficient to induce masculinization of females in exposed fish populations.

### 5.5.3. Conclusions

The levels of estrogenic and androgenic activity in treated municipal sewage from 15 different plants in south Queensland (Australia) and Canterbury (New Zealand) were below those reported by researchers in the United Kingdom. Trickling-filter technology is widely used in the UK (Angus et al. 2002), while activated sludge systems are more common in Australia. The lower levels of estrogenic and androgenic activities in treated sewage may be due to this difference in treatment technology. Furthermore, lower population densities in Australia and New Zealand may also result in lower loads of estrogenic and androgenic chemicals being released into the environment. Additional studies are needed to examine the potential long-term effects of exposure to treated sewage containing trace concentrations of estrogenic and androgenic chemicals on exposed wildlife, as well as to determine if the unique Australian and New Zealand fauna is more susceptible to hormonally active chemicals.

## 5.6. Co-author contributions

This chapter has been submitted to Ecotoxicology and Environmental Safety. Besides supervisory committee members, the co-author for this paper is Mike van den Heuvel (Forest Research, Rotorua, New Zealand). Mike provided logistical support for the work with the AR binding assay, including the necessary facilities and supplies.

# 6. Gonadal histopathology and anal fin morphology in *Gambusia holbrooki* exposed to treated municipal sewage effluent

## 6.1. Abstract

Feral mosquitofish living in undiluted treated municipal sewage in Queensland, Australia, were examined for morphological abnormalities indicative of reproductive dysfunction. Male and female mosquitofish were captured at two sites receiving undiluted treated sewage and compared with those captured at a reference site. Several morphological endpoints were examined, including length of the 4$^{th}$ and 6$^{th}$ anal fin rays and gonadal histology. Both males and females at one site exhibited minor elongation of the 4$^{th}$ anal fin ray consistent with exposure to androgenic stimulation, although the spatial extent of the effect was limited and not significant at other sites further downstream. No incidences of intersex were found. These findings suggest that the level of treatment of domestic sewage in Queensland is adequate to prevent significant reproductive abnormalities in exposed mosquitofish populations.

## 6.2. Introduction

Reproductive abnormalities have been reported in wild fish (Jobling and Tyler 2003) in estuarine and coastal waters in the United Kingdom (Jobling et al. 1998, Allen et al. 1999, van Aerle et al. 2001), continental Europe (Viganò et al. 2001), North America (Folmar et al. 2001), and Japan (Hashimoto et al. 2000). These abnormalities ranged from unusual plasma steroid levels and high levels of Vtg (the protein precursor of egg yolk) in males to morphological abnormalities of reproductive organs such as ovotestis, a pathological condition where gonads of gonochoristic fish contain both male and female sex cells ("intersex") (Purdom et al. 1994, Jobling et al. 1998). Such changes have been linked to exposure of the fish to hormonally active chemicals (termed endocrine-disrupting compounds, EDCs) present in treated sewage water discharges (Harries et al. 1996, 1999, Routledge et al. 1998, Rodgers-Gray et al. 2001, Christiansen et al. 2002). While it is still unclear whether

these organism-level abnormalities as a result of exposure to EDCs are translated into population-level effects, abnormal gonadal and hormonal changes in wild fish are undeniably a significant environmental issue and more research is needed to determine the geographical extent of endocrine disruption in wild fish (Jobling and Tyler 2003).

Eastern mosquitofish (*Gambusia holbrooki*, Girard 1859) are sexually dimorphic. Males are much smaller than the females and have an elongated anal fin, the gonopodium, which is used as an intromittent organ during copulation. Gonopodium development is under androgenic stimulation from the testis in the final stages of sexual maturation (Turner 1941), and can be inhibited by castration (Turner 1947) and to a lesser extent by exposure to estrogenic chemicals (17β-estradiol, 100 ng/L) (Doyle and Lim 2002). Conversely, laboratory exposure of juvenile females to androgenic stimulation (11-ketotestosterone, 20 μg/g) results in gonopodium-like elongation of the anal fin typical of juvenile males (Angus et al. 2001). Several field studies downstream of pulp and paper mills have shown a high incidence of masculinized females, indicating that chemicals with androgenic activity are present in the mill effluents (Bortone and Davis 1994, Bortone and Cody 1999, Parks et al. 2001). This hormone-dependent morphological attribute, along with their restricted home range, abundance, and widespread distribution in Australia, makes the mosquitofish a valuable local indicator species for exposure to EDCs (Bortone and Davis 1994, Overstreet et al. 1996). Gonopodium elongation in mosquitofish has been used as an endpoint for endocrine impacts in mosquitofish exposed to sewage water in Australia (Batty and Lim 1999). In that study, wild male *G. holbrooki* sampled in an industrial area downstream from a sewage and wastewater treatment plant in New South Wales had significantly shorter gonopodia than mosquitofish sampled at a reference site, suggesting the presence of estrogenic chemicals (Batty and Lim 1999). In a recent study (Leusch et al. Submitted, Chapter 5), we measured the estrogenicity and androgenicity in domestic sewage in southeast Queensland using a combination of chemical extraction techniques and *in vitro* bioassays. The present study is an extension of that work to examine possible environmental impacts of treated domestic sewage on exposed fish.

This study sought to determine if treated domestic sewage in Queensland, Australia, caused changes in gonopodium morphology or gonadal histology in exposed mosquitofish. To that end, the anal fin morphology and gonads of feral mosquitofish living in undiluted treated sewage from a small and a large domestic sewage treatment plant (STPs) were compared with mosquitofish sampled at a reference site.

## 6.3. Materials and Methods

### 6.3.1. Sampling and study sites

*G. holbrooki* were sampled in July 2003 at two test sites (sites A and B) near Brisbane in south Queensland, Australia. A reference site was also sampled and consisted of a wetland constructed for educational and recreational uses that receives water from a water storage reservoir located in a mostly forested catchment. Sewage effluent site A is an artificial wetland that provides tertiary treatment for a small municipal STP, and receives an average of 0.57 ML/d of secondary-treated sewage (plant F1A in Table 5.1). Three cells were sampled at site A: A1, A2, and A3. Secondary-treated sewage enters the wetland in cell A1 and is further treated as it slowly flows into cells A2 and then A3. The tertiary-treated sewage in cell A3 is then discharged into the environment (Table 6.1). Water residence time in the wetland is approximately 14 d. Sewage effluent site B is a holding pond for a much larger municipal STP and receives approximately 26 ML/d of secondary-treated sewage (Table 6.1; plant S11A in Table 5.1). Average monthly rainfall and air temperature in the Brisbane area for that year are reported in Fig. 6.1, while minimum and maximum water temperature during the month prior to sampling are reported in Table 6.1. Mosquitofish were captured in minnow traps baited with bread or dry dog food, transported to the laboratory in aerated water from the site, and processed within one week.

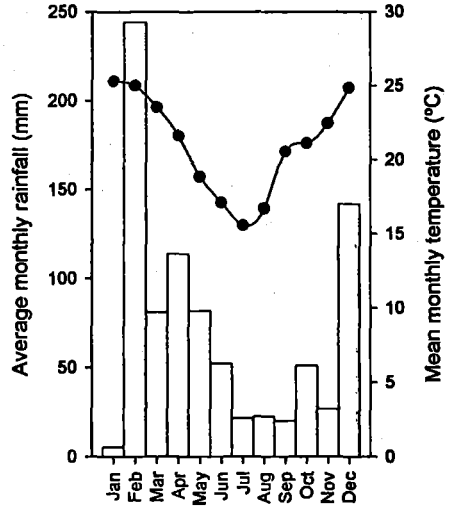


**Figure 6.1:** Average monthly temperature (connected circles) and rainfall (open bars) in Brisbane, Queensland, Australia. Data from Commonwealth Bureau of Meteorology, Brisbane, Qld, Australia. Sampling for this study took place in July.

**Table 6.1:** Description of the sampling sites, including minimum and maximum water temperature for one month prior to sampling.

| Site | | Water type | Water temp (°C) | STP Train* | Flow (ML/d) |
|---|---|---|---|---|---|
| Ref | | Water from dam reservoir | 16 – 19 | Not applicable | NA |
| A | A1 | Secondary-treated sewage | 13 – 19 | S, GGC, PS, **TF**, | 26 |
| | A2 | Partly tertiary-treated sewage from A1 | | SS, CC, **Wt** | |
| | A3 | Tertiary-treated sewage from A2 | | | |
| B | | Secondary-treated sewage | 15 – 20 | S, GGC, PS, **AS**, SS, CC, **HP** | 0.57 |

NA = not available.

* Letters in bold highlight differences between the two sewage treatment plants.

Steps of the treatment train: S = screens; GGC = grit and grease chamber; PS = primary settling tank; TF = trickling filter; AS = activated sludge bioreactor; SS = secondary settling tank; CC = chlorine contact tanks; Wt = wetland; HP = holding pond.

### 6.3.2. Morphometrics

Mosquitofish were anaesthetized in carbonated water and killed by spinal severance. Body length was measured from snout to caudal peduncle (standard length) to the nearest 0.1 mm using callipers, and the anal fin photographed using a Nikon Coolpix 4500 digital camera mounted on a Leica MZ6 stereomicroscope. The 4$^{th}$ and 6$^{th}$ anal fin rays were measured from base to tip to the nearest 0.01 mm from these images using Image Tool 3.0 (University of Texas Health Sciences Center in San Antonio, TX, USA). Three endpoints were used to express anal fin length in this study: (1) the length of the 4$^{th}$ anal fin ray (GP4) as in Toft et al. (2003); (2) the difference in length between the 4$^{th}$ and 6$^{th}$ anal fin ray (GPx) as in Batty and Lim (1999); and (3) the ratio of the length of the 4$^{th}$ to the 6$^{th}$ anal fin ray (4:6 ratio) as in Angus et al. (2001). Mosquitofish were assigned a phenotypic sex and age group based on anal fin morphology. Fish exhibiting elongation or widening of the base of the 3$^{rd}$ anal fin ray but incomplete gonopodial development were phenotypic juvenile males. Fish with a fully developed gonopodium (determined by the presence of terminal hooks and elbow) were classed as adult males. Fish showing no elongation or widening of the base of the 3$^{rd}$ anal fin

ray and of <20 mm in length were assigned to the juvenile female group, and those >20 mm long (inclusive) were assigned to the adult female phenotype.

### 6.3.3. Gonadal histopathology

Based on their phenotype, 19 mosquitofish were selected at each site for gonadal histology: two juvenile males, ten adult males, two juvenile females, and five adult females. Their gonads were excised and fixed in 200 µL of 4% glutaraldehyde in 0.1 M phosphate buffer (5.99 g $NaH_2PO_4$, 13.4 g Na2HPO4-7H2O, pH 7.2) for 24h. Gonads were then transferred to 30% sucrose in 0.1 M phosphate buffer and stored at 4°C for at least 24h until processed. The gonads were embedded in optimal cutting temperature (OCT) compound (ProSciTech, Thuringowa, Qld, Australia), and a combination of longitudinal or transverse sequential 6-µm-thick sections (Bernet et al. 1999) were made at –17°C in a cryostat (IEC Minotome Plus). The sections were mounted on frosted slides (Superfrost Plus, Menzel-Gläser, Braunschweig, Germany) and stained with Mayer's hematoxylin and eosin, dehydrated and mounted with DPX (ProSciTech, Thuringowa, Qld, Australia). Sections were examined under light microscopy and photographed. Gonadal structures were identified based on Patiño (1995) and Nolan et al. (2001).

### 6.3.4. Statistical analysis

All statistical tests were performed with SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA) except the $\chi^2$ test, which was performed by hand and tabulated with Excel9 (Microsoft Corp., Seattle, WA, USA). Significance was set at $\alpha = 0.05$ for all tests. A $\chi^2$ test was used to test if the distribution of mosquitofish at the test sites was significantly different from that at the reference site. A one-way analysis of variance (ANOVA) was used to test for differences in standard body length among the different sites, followed by Bonferroni's test for multiple comparisons. With the females and the adult males, GP4 and GPx were compared among the different sampling sites using an analysis of covariance (ANCOVA) with standard body length as the covariate to correct for differences in body length among different sites, followed by Bonferroni's test for multiple comparisons. Gonopodium elongation ratio (4:6 ratio) was also compared among sampling sites using a one-way

ANOVA, followed by Bonferroni's test. Because the previous Australian study (Batty and Lim 1999) divided mosquitofish into size classes instead of using an ANCOVA, we also used this method to analyze our results. Adult males were divided into three different groups based on standard length, and the three endpoints (GP4, GPx, and 4:6 ratio) compared among different sampling sites within a size class using a one-way ANOVA followed by Bonferroni's test.

## 6.4. Results

### 6.4.1. Standard length and phenotypic distribution of captured mosquitofish

A total of 694 mosquitofish were captured and examined (Table 6.2). There were significant differences in standard body length of the sampled mosquitofish among the different sites (Table 6.3). Overall, the phenotypic distribution by age and sex class at site B was not significantly different from that of the reference site, but it was significantly different between the reference site and all A sub-sites ($\chi^2$ test, $p < 0.05$, Table 6.2). At all three A sites, there were significantly fewer adult males and more adult females than expected if the population distribution had been the same as at the reference site ($\chi^2$ test, $p < 0.05$, Table 6.2). At site A2, there were significantly more juvenile males than expected, while at sites A1 and A3 there were fewer juvenile females than expected ($p < 0.05$, Table 6.2). With the exception of site A3 where most of the mosquitofish sampled were females (72.9%), the sex ratio at all other sites was slightly biased towards males, which made up 52 – 59% of the population. No gravid females were found at the reference site or at site B, but were present at site A and made up to 9% of the catch at A1. Juveniles (males and females combined) made up 17 – 36% of the total captured (Table 6.2).

**Table 6.2:** Proportion of mosquitofish sampled at a reference and two test sites in south Queensland, Australia, arranged by phenotype.

| Site | Juvenile male[a] | Adult male[b] | Juvenile female[c] | Adult female[d] | Total | Gravid female[e] |
|---|---|---|---|---|---|---|
| A1 | 0.12 (28) | 0.40 (94)* | 0.05 (12)** | 0.42 (99)** | 1.00 (233) | 0.09 (21) |
| A2 | 0.17 (43)** | 0.35 (88)** | 0.19 (48) | 0.29 (73)* | 1.00 (252) | 0.02 (4) |
| A3 | 0.13 (18) | 0.13 (18)** | 0.10 (13)* | 0.63 (84)** | 1.00 (133) | 0.01 (2) |
| Ref | 0.09 (2) | 0.50 (11) | 0.18 (4) | 0.23 (5) | 1.00 (22) | 0 |
| B | 0.15 (8) | 0.43 (23) | 0.18 (10) | 0.24 (13) | 1.00 (54) | 0 |

The number in bracket is the sample size (n). The asterisks indicate groups that are statistically different from the expected value based on the distribution at the reference site ($\chi^2$ test, * $p < 0.05$, ** $p < 0.01$).

[a] Elongation or widening of the base of the 3rd anal fin ray, but incomplete gonopodial development.

[b] Presence of a fully developed gonopodium with terminal hooks and elbow.

[c] Fish <20 mm (exclusive) standard length showing no elongation or widening of the base of the 3rd anal fin ray.

[d] Fish >20 mm (inclusive) standard length showing no elongation or widening of the base of the 3rd anal fin ray – includes gravid females.

[e] Identified by the presence of a gravid spot on the lower abdomen. Not included in the $\chi^2$ analysis.

**Table 6.3:** Standard length of mosquitofish captured at a reference and two test sites in south Queensland, Australia, arranged by phenotype.

| Site | Juvenile males[1] | Adult males[2] | Females[3] |
|---|---|---|---|
| A1 | 19.61 ± 0.31 (28) c | 20.45 ± 0.12 (92) b | 25.37 ± 0.39 (108) b |
| A2 | 19.15 ± 0.25 (42) bc | 19.58 ± 0.14 (88) ac | 21.03 ± 0.34 (104) a |
| A3 | 19.86 ± 0.40 (17) c | 20.43 ± 0.23 (18) bc | 24.58 ± 0.45 (75) b |
| Ref | 16.15 ± 0.85 (2) ab | 18.91 ± 0.42 (11) a | 19.53 ± 1.28 (9) a |
| B | 16.93 ± 0.45 (7) a | 21.76 ± 0.34 (23) d | 20.85 ± 0.78 (21) a |

The number in brackets is the sample size (n). Different letters indicate statistically different groups (one-way ANOVA $p < 0.001$ followed by Bonferroni's $p < 0.05$).

[1] Elongation or widening of the base of the 3rd anal fin, but incomplete gonopodial development.

[2] Presence of a fully developed gonopodium with terminal hooks and elbow.

[3] Showing no elongation or widening of the base of the 3rd anal fin.

### 6.4.2. Anal fin and gonopodium development

A typical female anal fin and male gonopodium are presented in Fig. 6.2. Typical juvenile female and male anal fins are shown in Fig. 6.3A and 6.3B, respectively. Juvenile males as small as 15 mm could be distinguished from juvenile females by the width of base of ray 3 (Fig. 6.3B) (Turner 1941), and in more advanced stages by the obvious elongation of rays 3, 4, and 5 (Fig. 6.3B and 6.3C). Different stages of gonopodium development were discernible (Fig. 6.3D–F).

### 6.4.3. Length of the 4$^{th}$ anal fin ray (GP4)

The length of the 4$^{th}$ anal fin ray (GP4) was significantly correlated with standard body length in females and adult males (linear regression ANOVA, $p < 0.01$ at all sites, Fig. 6.4) and there were no significant differences in the slopes of the linear regressions (ANCOVA, interaction $p = 0.989$ and $p = 0.845$ for females and adult males, respectively). There were significant differences in GP4 among the different sites with adult males but not with females (ANCOVA, $p = 0.001$ and $p = 0.054$, respectively, Fig. 6.5A and 6.5B). None of the sites were significantly different from the reference site (Bonferroni's, $p > 0.05$), but adult males at site A1 had significantly longer GP4 than those at sites A2 and B (Bonferroni's, $p = 0.006$ and $p = 0.022$, respectively, Fig. 6.5A and 6.5B). When adult males were separated into size classes, there were significant differences in GP4 only with the smallest adult males (one-way ANOVA, $p = 0.021$), where males at site A1 had significantly longer GP4 than those at the reference site and at site A2 (Bonferroni's, $p = 0.040$ and $p = 0.033$, respectively, Table 6.4).

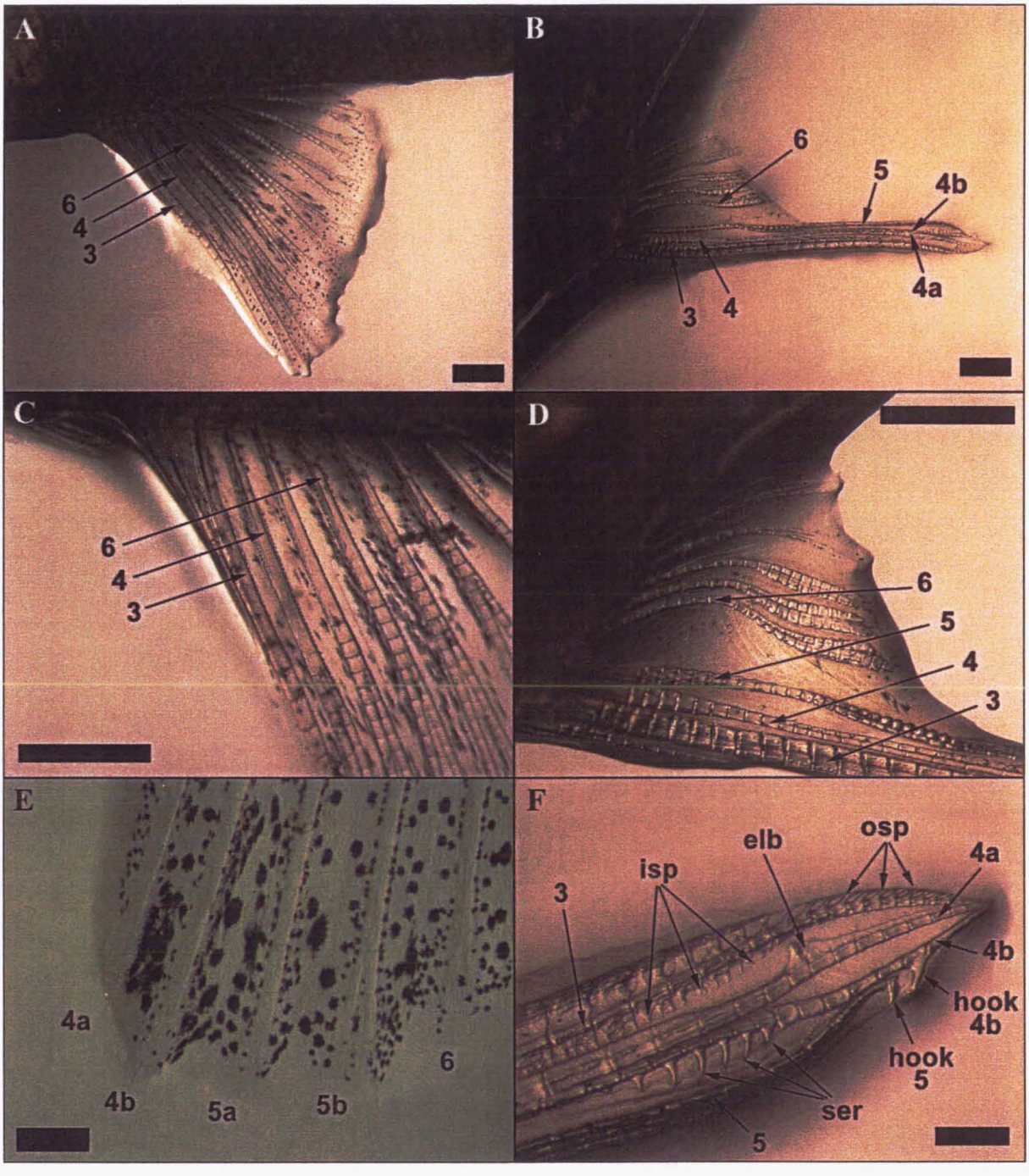


**Figure 6.2:** Anal fin of *G. holbrooki*. **A**: Adult female anal fin, with rays 3,4, and 6 labelled (bar = 1 mm). **B**: Adult male anal fin, with rays 3 to 6 labelled (bar = 1 mm). **C**: Close up of the base of the anal fin rays of an adult female, with rays 3, 4, and 6 labelled (bar = 1 mm). **D**: Close up of the base of the gonopodium of an adult male, with rays 3 to 6 labelled (bar = 1 mm). **E**: Tip of the anal fin rays of an adult female, with rays 4a to 6a labelled (bar = 0.2 mm). **F**: Tip of the gonopodium of an adult male, showing the serrae (ser) on ray 4b, the inner and outer spines (isp and osp, respectively) on ray 3, the hooks on ray 4b and 5, and the elbow (elb) or blade on ray 4a (bar = 0.2 mm).

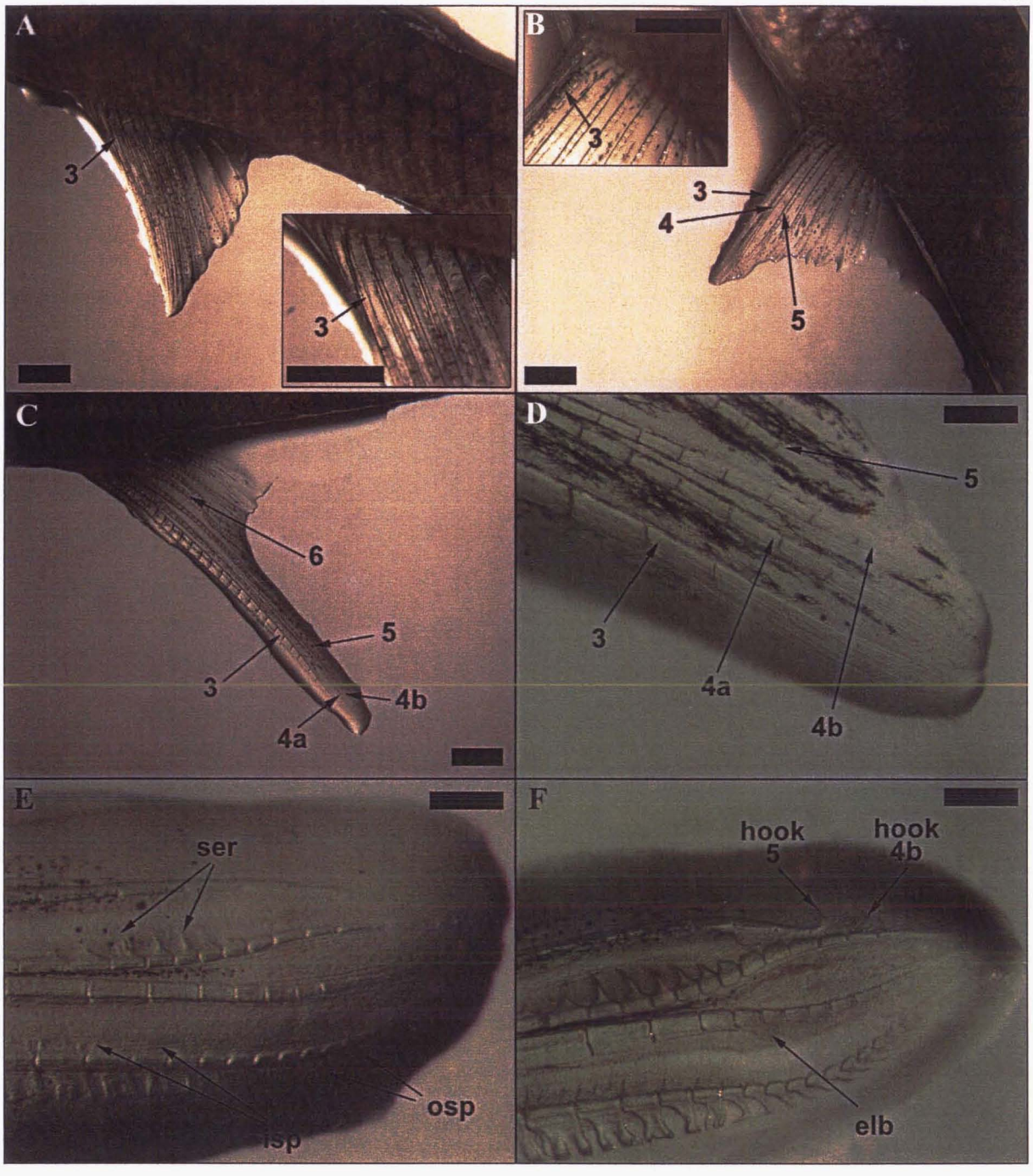


**Figure 6.3:** Anal fin of juvenile *G. holbrooki*, showing the development of the gonopodium. **A**: Juvenile female anal fin, inset: close-up of the base of the fin, showing the small width of ray 3 (bar = 1 mm). **B**: Juvenile male anal fin, inset: close-up of the base of the fin, showing the wider ray 3 (bar = 1 mm). **C**: Developing gonopodium of a juvenile male (bar = 1 mm). **D**: Tip of a developing gonopodium in stage 1 (bar = 0.2 mm). **E**: Tip of a developing gonopodium in stage 2, showing the developing serrae (ser) on ray 4b as well as the inner and outer spines (isp and osp, respectively) on ray 3 (bar = 0.2 mm). **F**: Tip of a developing gonopodium in stage 3, showing the developing hooks on ray 4b and 5 and the early elbow (elb) on ray 4a (bar = 0.2 mm).

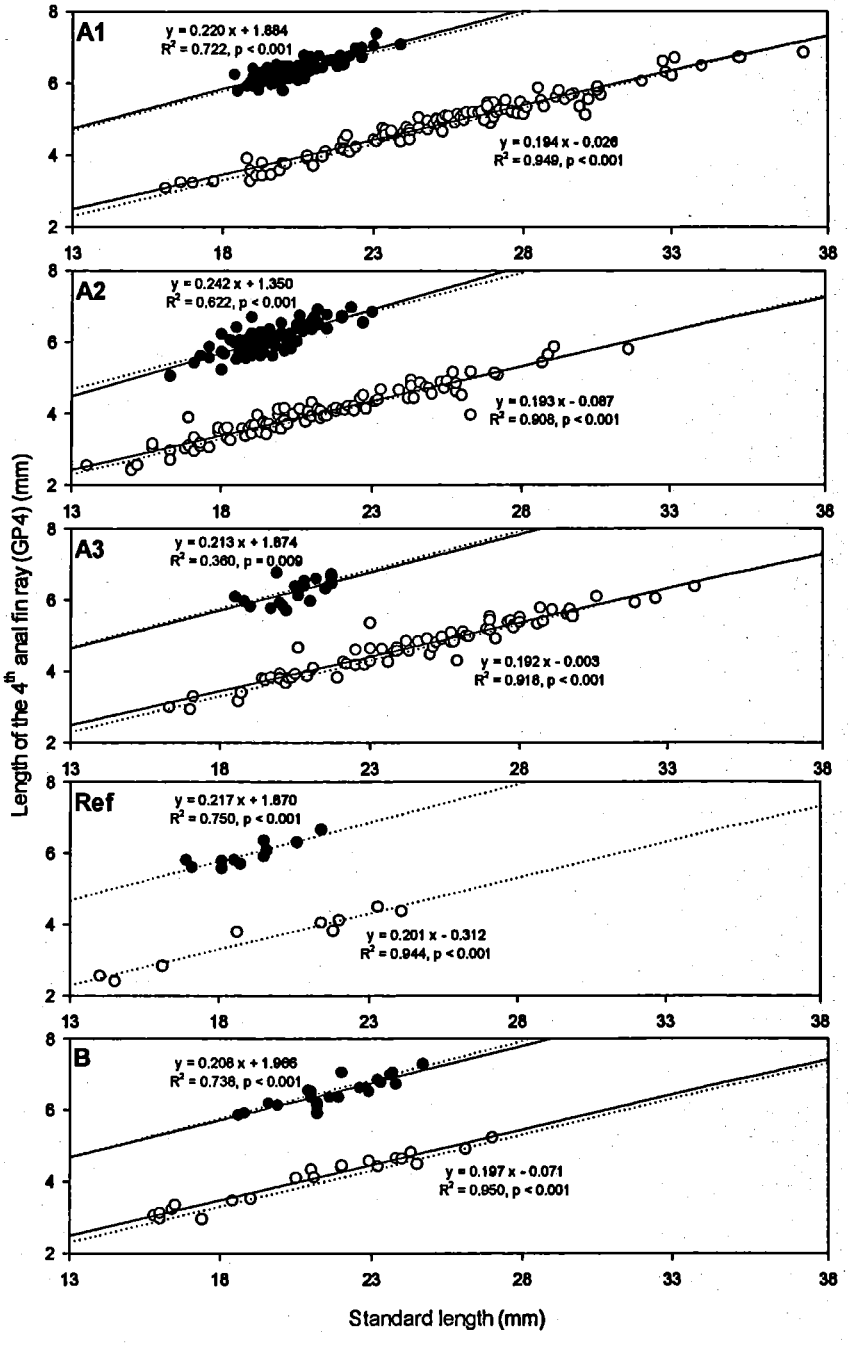


**Figure 6.4:** Length of the 4$^{th}$ anal fin ray (GP4) versus standard body length in female (O) and adult male (●) *G. holbrooki* collected at two sites receiving secondary-treated sewage (A and B) and a reference site. For each sex, the solid line is the regression for that site, and the dashed line is the regression for the reference site. The equation of the regression line for each sex is shown for each site, with its associated $R^2$ and p value.

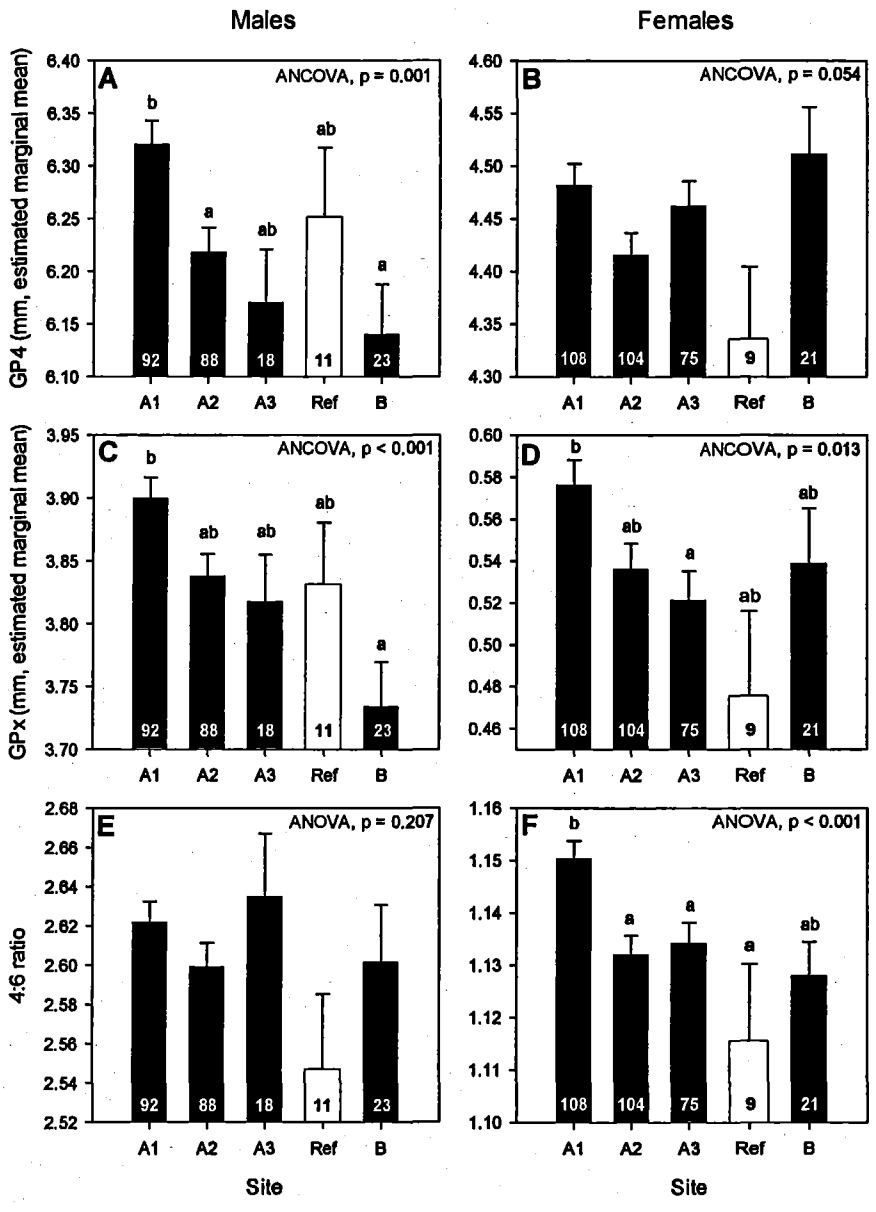


**Figure 6.5:** Length of the 4th anal fin ray (GP4), relative extension (GPx), and elongation ratio (4:6 ratio) in male (left) and female (right) *G. holbrooki* collected at two sites receiving secondary-treated sewage (A and B) and a reference site (Ref). Values for GP4 and GPx are estimated marginal means corrected for standard body length at each site ± SE, while 4:6 ratio is the mean at each site ± SE. The value at the bottom of each bar is the sample size. Different letters indicate statistically significant differences among different sites (for GP4 and GPx: one-way ANCOVA with standard body length as covariate followed by Bonferroni's test; for 4:6 ratio: one-way ANOVA followed by Bonferroni's test; $\alpha = 0.05$ for all tests).

**Table 6.4:** Length of the 4th anal fin ray (GP4), relative GP4 extension (GPx), and elongation ratio (4:6 ratio) for adult male mosquitofish arranged in three different size classes.

| | Size class | | |
|---|---|---|---|
| **Site** | **16.3 - 19.1mm** | **19.2 - 21.9mm** | **22.0 - 24.7mm** |
| *GP4 (mm)* | | | |
| A1 | 6.11 ± 0.05 (13) b | 6.36 ± 0.03 (69) | 6.90 ± 0.08 (10) |
| A2 | 5.85 ± 0.05 (37) a | 6.19 ± 0.05 (45) | 6.72 ± 0.07 (6) |
| A3 | 5.96 ± 0.08 (3) ab | 6.28 ± 0.09 (15) | NA |
| Reference | 5.72 ± 0.04 (6) a | 6.26 ± 0.13 (5) | NA |
| B | 5.89 ± 0.03 (2) ab | 6.28 ± 0.06 (11) | 6.86 ± 0.08 (10) |
| *GPx (mm)* | | | |
| A1 | 3.75 ± 0.04 (13) b | 3.93 ± 0.02 (69) | 4.30 ± 0.07 (10) |
| A2 | 3.57 ± 0.03 (37) a | 3.81 ± 0.04 (45) | 4.20 ± 0.04 (6) |
| A3 | 3.63 ± 0.07 (3) ab | 3.91 ± 0.07 (15) | NA |
| Reference | 3.44 ± 0.04 (6) a | 3.84 ± 0.14 (5) | NA |
| B | 3.60 ± 0.01 (2) ab | 3.81 ± 0.04 (11) | 4.29 ± 0.07 (10) |
| *4:6 ratio* | | | |
| A1 | 2.59 ± 0.02 (13) | 2.62 ± 0.01 (69) | 2.66 ± 0.03 (10) |
| A2 | 2.58 ± 0.02 (37) | 2.61 ± 0.02 (45) | 2.67 ± 0.03 (6) |
| A3 | 2.56 ± 0.04 (3) | 2.65 ± 0.04 (15) | NA |
| Reference | 2.51 ± 0.04 (6) | 2.59 ± 0.07 (5) | NA |
| B | 2.57 ± 0.01 (2) | 2.55 ± 0.04 (11) | 2.67 ± 0.04 (10) |

Values are means at a site within a size class ± SE. The value in brackets is the sample size. Different letters indicate statistically significant differences among sites within that size class (one-way ANOVA followed by Bonferroni's test, $\alpha = 0.05$).

### 6.4.4. Relative GP4 extension (GPx)

GPx was significantly correlated with standard body length in females and adult males (linear regression ANOVA, $p < 0.01$ at all sites) and there were no significant differences in the slopes of the linear regressions (ANCOVA, interaction $p = 0.276$ and $p = 0.965$ for females and adult males, respectively). There were significant differences in GPx of mosquitofish among the different sites in both adult males and females (ANCOVA, $p < 0.001$ and $p = 0.013$, respectively, Fig. 6.5C and 6.5D). None of the mosquitofish at any of the sites had significantly different GPx compared with those at the reference site (Bonferroni's, $p > 0.05$), but adult males at site A1 had significantly greater GPx than those at site B, while females at site A1 had significantly greater GPx than those at site A3 (Bonferroni's, $p < 0.001$ and $p = 0.025$, respectively, Fig. 6.5C and 6.5D). When adult males were separated into size classes, significant differences in GPx were seen only in the smallest individuals (one-way ANOVA, $p = 0.010$), where males at site A1 had significantly greater GPx than those at the reference site and at site A2 (Bonferroni's, $p = 0.008$ and $p = 0.042$, respectively, Table 6.4).

### 6.4.5. Elongation ratio (4:6 ratio)

There were significant differences in 4:6 ratio among the different sites with females, but not with adult males (one-way ANOVA, $p < 0.001$ and $p = 0.207$, respectively, Fig. 6.5E and 6.5F). Females at site A1 had significantly higher 4:6 ratio than those at sites A2, A3, and the reference site (Bonferroni's, $p = 0.002$, $p = 0.029$, and $p = 0.045$, respectively). When adult males were separated into size classes, there were no significant differences in 4:6 ratio among sites with any of the size classes (one-way ANOVA, $p > 0.20$ for all size classes, Table 6.4). The frequency distribution was clearly bimodal, with a very thin peak at 1.1 – 1.2 from the females and a second wider peak at either 2.4 – 2.5 or 2.5 – 2.6 from the adult males (Fig. 6.6).

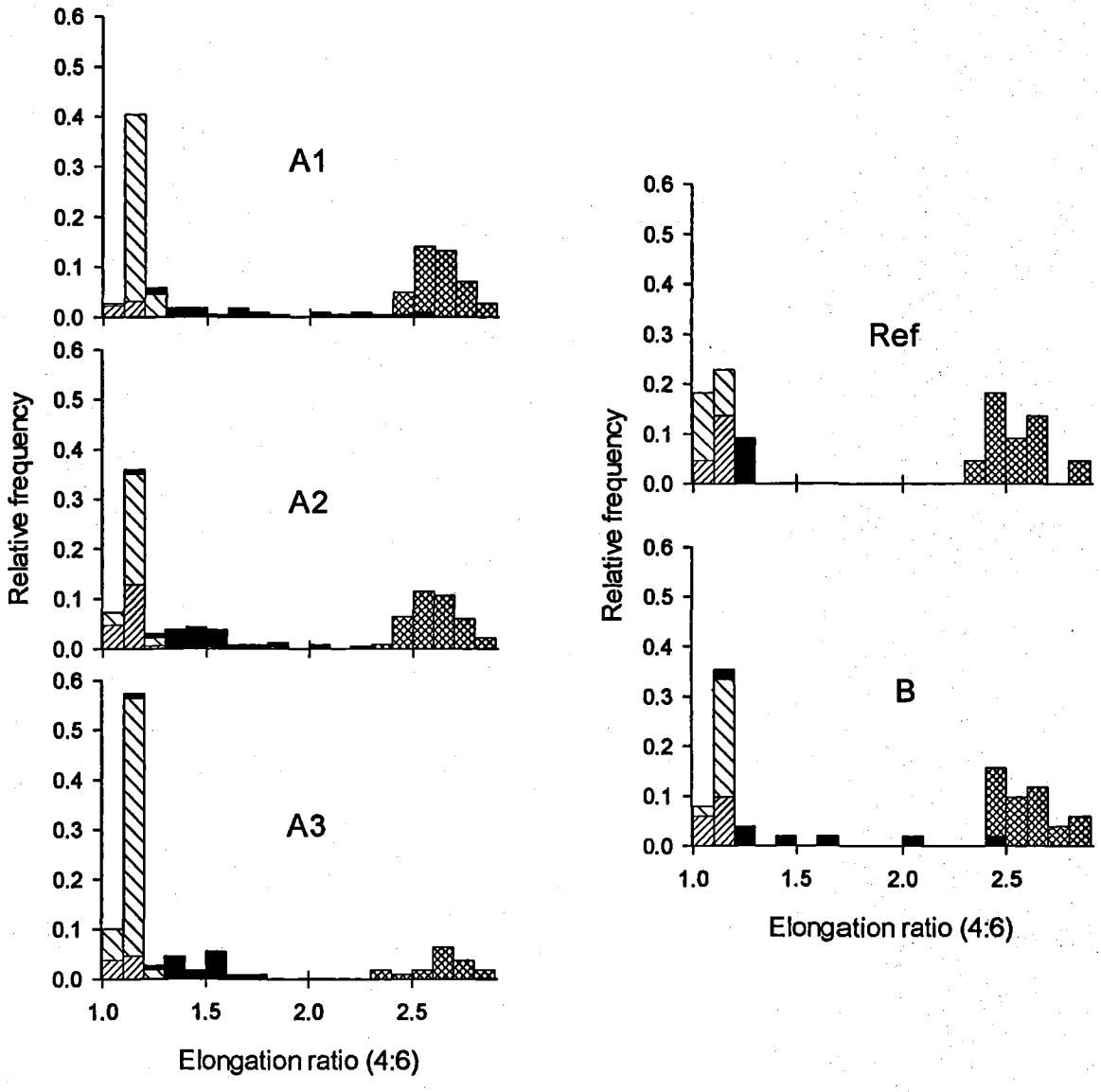


**Figure 6.6:** Relative frequency distribution of the gonopodium elongation ratio (ratio of 4th to 6th anal fin ray) of mosquitofish collected at two sites receiving secondary-treated sewage (A and B) and a reference site (Ref). Data are organized in equal intervals of 0.1 units from 1.0 to 2.9. Legend: ▨ juvenile females; ▧ adult females; ■ juvenile males; ▩ adult males.

### 6.4.6. Gonadal histopathology

There was no evidence of ovotestis ("intersex") in mosquitofish at any of the sites sampled, and no gross morphological differences between fish from test and reference sites. Testes of juvenile males (males with undeveloped or developing gonopodia) were in early stages of testicular development containing spermatocysts with spermatocytes or spermatids (Fig. 6.7B). Testes from adult males with fully developed gonopodia contained spermatocysts in all stages of development (Fig. 6.7A, 6.7C, and 6.7D), as well as spermatozeugmata in the lumen of the efferent duct (Fig. 6.7A and 6.7E). In female gonads, several stages of follicular development were visible (Fig. 6.8A), illustrating the asynchronous nature of ovaries of *G. holbrooki*. Similarly to males, ovaries of adult females (Fig. 6.8D and 6.8E) were in more advanced stages of development than those of juvenile females (Fig. 6.8C).

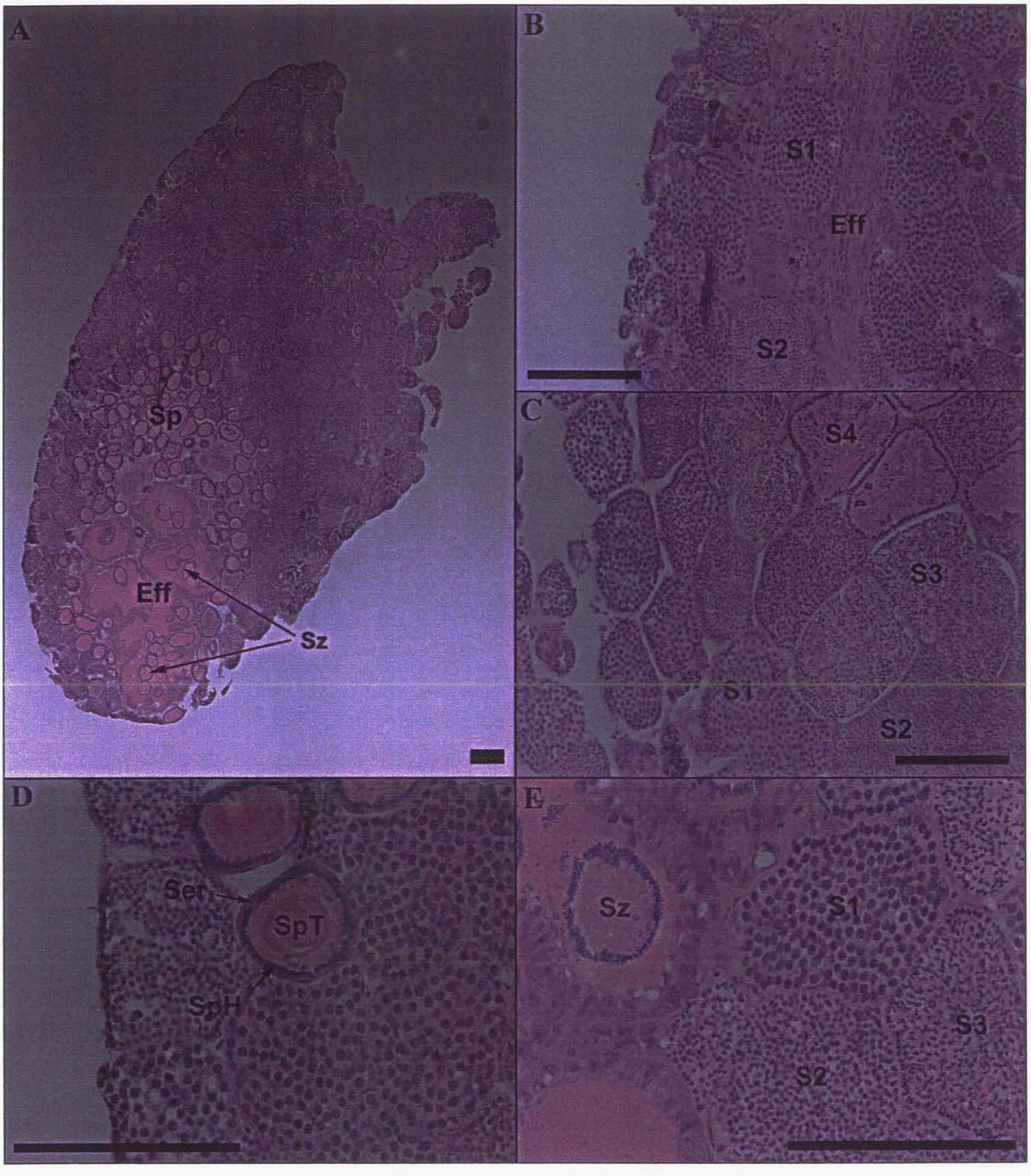


**Figure 6.7:** Gonadal histology of male *G. holbrooki*. Bar = 100μm. **A**: Transverse section of adult male gonad showing spermatocysts in early stages of development at the periphery and fully developed sperm (Sp) near the efferent duct (Eff). Spermatozeugmata (Sz) are visible in the lumen of the efferent duct. **B**: Longitudinal section of juvenile male gonad, showing spermatocysts in early stages of development (spermatocytes S1 and spermatids S2) and the efferent duct (Eff). **C**: Stages of spermatocyst development in adult male, from spermatocytes (S1), to spermatids (S2), to early (S3) and late stages of spermiogenesis (S4). **D**: Detail of a spermatocyst in the late development, showing sperm tails (SpT) in the centre and sperm heads (SpH) embedded in the Sertoli (Ser) cell barrier. **E**: Detail of the efferent duct, showing spermatocysts in varied stages of development (S1 – S3) and a spermatozeugmata (Sz) in the lumen.

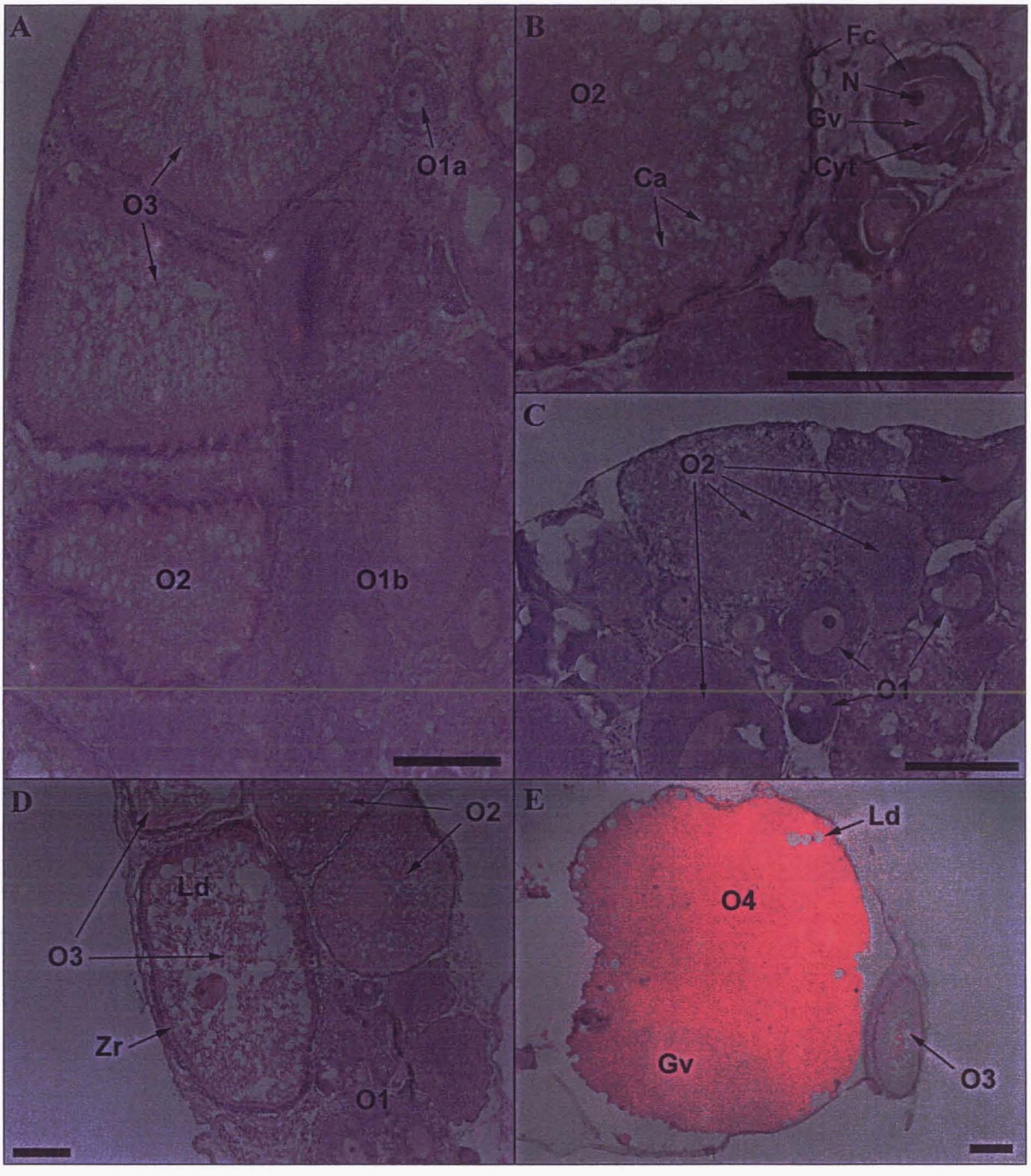


**Figure 6.8:** Gonadal histology of female *G. holbrooki*. Bar = 100μm. **A**: Adult female gonad, showing several stages of follicular development, from early (O1a) to late primary oocytes (O1b), to cortical alveoli oocytes (O2), to early vitellogenic oocytes (O3). **B**: Primary oocyte structure, showing the germinal vesicle (Gv), the nucleolus (N), and the cytoplasm (Cyt) surrounded by follicular cells (Fc). On the left, cortical alveoli (Ca) of a cortical alveoli oocyte (O2) are visible. **C**: Juvenile female gonad, showing primary (O1) and early and late cortical alveoli oocytes (O2). **D**: Adult female gonad, showing primary oocytes (O1), cortical alveoli oocytes (O2), and early vitellogenic oocytes (O3). The zona radiata (Zr) and lipid droplets (Ld) are discernible in the vitellogenic oocyte. **E**: Adult female gonad showing an early vitellogenic (O3) and maturing oocytes (O4). The germinal vesicle (Gv) in O4 has almost disappeared and lipid droplets (Ld) have coalesced at the periphery of the oocyte.

## 6.5. Discussion

In the present study, eastern mosquitofish (*G. holbrooki*) living in undiluted treated domestic sewage in South Queensland exhibited minor morphological differences consistent with androgenic stimulation, while mosquitofish downstream of another STP showed no statistically significant signs of either estrogenic or androgenic effects compared with reference fish. Adult male mosquitofish sampled immediately downstream of the effluent discharge of STP A (site A1) had the longest gonopodia of all mosquitofish sampled (Fig. 6.5), and gonopodia of small adult males were significantly longer than those captured at the reference site (Table 6.4). This is similar to the results of Angus et al. (2002) who found that western mosquitofish (*G. affinis*) captured downstream of an STP had significantly elongated gonopodia and larger testes (when corrected for total body weight) than those at a reference site, both of which are suggestive of an androgenic effect. Site A1, however, also had the highest number of gravid females, with almost a quarter of all the adult females captured at that site gravid (Table 6.2). A recent study in New Zealand reported a significant stimulation of reproductive function in rainbow trout exposed to secondary-treated sewage, most likely due to the presence of low levels of estrogenic hormones (Höger et al. in press). The proportion of gravid females in the sample decreased dramatically at the downstream sites (A2 and A3) where less than 2% of the adult female population was gravid (Table 6.2), suggesting that this putative estrogenic effect was associated with short-lived chemicals. This illustrates the complexities in understanding and extrapolating the effects of complex mixtures, where both androgenic and estrogenic chemicals may be present, on whole organisms where androgens may be metabolized into estrogens by endogenous enzymes. For example, aromatase is an enzyme involved in steroidogenesis that converts the androgen testosterone to the estrogen 17β-estradiol. Nevertheless, based on anal fin morphology, there appears to be evidence of an androgenic effect at site A1.

The treated domestic sewage water discharged from the sewage treatment plant at site B (STP B) did not significantly affect any of the endpoints used in this study. STP B treated municipal sewage from a much larger community (100,000 people-equivalents vs only 2200 at STP A) and, while the majority of the influent was domestic sewage, it is likely to also receive some industrial and commercial waste that would dilute the potent hormones present in domestic sewage. In fact, the results of this study suggest that concentrations of androgenic chemicals were higher in effluent from STP A than from STP B. Secondary

treatment at STP A relies on a trickling filter, while activated sludge treatment was in use at STP B. Preliminary results of a survey of 13 STPs in Queensland, using solid-phase extraction and *in vitro* bioassays, suggest that fixed-film systems (such as a trickling filter) are much less efficient at removing androgenic and estrogenic activity from sewage water than suspended-film systems (such as activated sludge) (Leusch, unpublished results; Chapter 5). In that study, the estrogenicity of the effluent from STP A was approximately 6 – 7 ng/L of 17β-estradiol equivalent, while androgenicity was approximately 2 μg/L of testosterone equivalent, the highest of secondary-treated effluents tested (estrogenicity of the effluent at STP B was less than 4 ng/L of 17β-estradiol equivalents; androgenicity was not measured). The wetland of site A from which the mosquitofish were sampled, however, is a part of the treatment train of STP A. A closer look at sites A2 and A3 reveals a trend towards normalization of gonopodium morphology downstream from site A1, and the anal fins of mosquitofish captured at these sites did not differ significantly from those of fish at the reference site (Table 6.4 and Fig. 6.5). This suggests that the wetland efficiently removed the androgenic activity still present in the secondary-treated effluent and that by the time the wastewater has flowed through the entire wetland (approximately 14 d), the activity has been significantly lowered to a point where it was no longer detectable using the mosquitofish morphological biomarkers.

The sample size at the reference site was comparatively low despite our best efforts, with only 22 mosquitofish caught compared with more than 100 at all A sites. The wetland at site A was much shallower than the reference site, which meant the openings of the minnow traps were only a few centimetres below the surface, ideal for capturing mosquitofish in the early morning hours. Site B was relatively deep, but the sheer number of mosquitofish present yielded a good catch. The small sample size at the reference site and the associated relatively higher standard error did not allow more concrete conclusions to be made. One way to tentatively resolve this issue was to use site A3 (the most downstream and least-impacted of the A sites) as a reference surrogate, a scenario that led to the same conclusions with site A1 being significantly different from all other sites (Fig. 6.5).

In this study, mosquitofish were assigned a phenotypic sex and age class based on anal fin morphology (Table 6.2). This phenotypic distribution could also be reflecting the effects of hormonal disruptors. For example, juvenile females exposed to androgenic chemicals may exhibit elongated anal fins characteristic of juvenile males (Angus et al. 2001). Particular attention was therefore paid to those fish classified as juvenile males, but only site A2 had

significantly more fish with that phenotype than would have been expected from the distribution at the reference site (17.1% vs 9.1%). Again, the limited sample size from the reference site imposed restrictions on the conclusions to be drawn. For example, just one extra juvenile male at the reference site would have increased their relative proportion to 13% and the difference with site A2 would then not have been statistically significant. The proportion of mosquitofish with the juvenile male phenotype, however, was fairly similar at all test sites, ranging from 12.0% at A1 to 17.1% at A2 (Table 6.2).

Furthermore, gonadal sex (determined histologically for a randomly selected group of mosquitofish) was always consistent with the assigned phenotypic sex, suggesting that the higher proportion of the juvenile male phenotype at site A2 was not a reflection of arrhenoidy. Also of interest in the phenotypic sex and age class distribution is the high proportion of adult females captured from site A3 (63.2% vs 42.5% and 29.0% at A1 and A2, respectively). Due to the subtle nature of the effects on the presumably more sensitive gonopodium development, this is thought to be an artefact of sampling rather than an actual endocrine effect on sex determination. This was reinforced by the lack of intersex gonads in any of the sampled fish. It might be an indication that female mosquitofish preferentially group with other females; or a sampling artefact. Either way, more research is needed to explain this phenomenon.

Morphological examination of adult male gonopodia, and in particular the presence of inner spines on ray 3 of the gonopodium (isp, Fig. 6.2F), clearly identified the species of the sampled fish as *holbrooki*, since the western mosquitofish (*G. affinis*) does not exhibit these (Rauchenberger 1989). However, the 4:6 ratio of male and female *G. holbrooki* reported in this study was very similar to those reported for *G. affinis* (Angus et al. 2001), and gonopodium development in *G. holbrooki* appeared to follow the same sequence as that previously described for *G. affinis* (Turner 1941, Angus et al. 2001), starting with widening of the base of ray 3 (stage 1, Fig. 6.3B), followed by the elongation of rays 3, 4, and 5 (stage 2, Fig. 6.3C and 6.3D), development of spines on ray 3 and serrae on ray 4a (stage 3, Fig. 6.3E), and finally, development of hooks on rays 4b and 5 and of the elbow (or blade) on ray 4a (final stage, Fig. 6.3F).

Although mosquitofish have been shown to be a relevant organism in which to measure exposure to hormonally active chemicals in wildlife (Bortone and Davis 1994, Batty and Lim 1999), it is possible that the morphological endpoints used in this study were not sensitive enough to detect more subtle effects. For example, histological analyses of the gonads

revealed no apparent differences between the test and reference sites, and no incidences of ovotestis were observed in mosquitofish from any of the sampled sites. However, few cases of ovotestis in this species have been reported in the literature, and it is possible that gonadal differentiation in *Gambusia* is not sensitive enough to be significantly affected by environmentally relevant concentrations of androgens and estrogens that only appear to inhibit sexual development (Angus et al. 2002). Laboratory studies show significant effects of androgenic and estrogenic chemicals on gonopodium development, but the concentrations used were relatively high (Angus et al. 2001, Doyle and Lim 2002). More research needs to be done to examine the effect of very low levels of hormonally active chemicals on those morphological endpoints, as well as in the development of more sensitive biomarkers. We are in the process of validating a method to measure Vtg mRNA in mosquitofish using reverse transcription real-time polymerase chain reaction, which may provide a more sensitive indicator of exposure to estrogenic chemicals.

In conclusion, undiluted tertiary-treated domestic effluent from two Queensland STPs had no measurable effect on morphological biomarkers in mosquitofish, indicating that endocrine disruption may not be a significant issue associated with *domestic* treated sewage water in Australia. The treatment plants monitored in this study dealt almost exclusively with sewage, but it is common for STPs to also treat industrial wastewater. Batty and Lim (1999) showed that mosquitofish living in waterways receiving industrial wastewater in New South Wales exhibited signs consistent with exposure to estrogenic chemicals; therefore, more field surveys and fundamental mechanistic studies are needed to provide a more complete picture of the endocrine-disrupting potential of STP discharges in Australasia. The issue of endocrine disruption is further complicated by the role of endogenous enzymes (e.g., aromatase). More research into the mechanisms of endocrine disruption in whole organisms is needed if field results are to be sensibly interpreted and understood.

## 6.6. Co-author contribution

This chapter has been submitted to Archives of Environmental Toxicology and Chemistry. Besides supervisory committee members, the co-author for this paper is Graham Kay (Agriculture and Life Sciences, Lincoln University, New Zealand). Graham examined some of the histology slides for subtle changes in gonadal histology.

# 7. Quantification of vitellogenin mRNA induction in mosquitofish (*Gambusia affinis*) by reverse transcription real-time polymerase chain reaction (RT-PCR)

## 7.1. Abstract

A method to quantify induction of vitellogenin (Vtg) mRNA in adult male mosquitofish was developed. Male mosquitofish were exposed to 0, 1, 20, and 250 ng/L of 17β-estradiol (E2) for 4 and 8 d in static exposures, and liver Vtg mRNA and 18S rRNA expression were quantified in duplex RT-PCR. Liver 18S rRNA expression was very consistent among individuals, and there was a highly significant increase in Vtg mRNA expression after exposure of mosquitofish for just 4 d at 250 ng/L of E2. Lower doses did not induce Vtg mRNA expression even at 4 or 8 d. This method could be used as a rapid test to detect exposure of mosquitofish to oestrogenic chemicals. Further work is needed to determine if increased Vtg mRNA levels in male mosquitofish induce Vtg synthesis, and to determine the usefulness of the method in field sampling.

## 7.2. Introduction

Vitellogenin (Vtg) is a glycolipophosphoprotein precursor to egg yolk produced in the liver of mature female fish under estrogenic stimulation. Although it is only detected at very low levels in males under normal conditions, Vtg expression can be greatly induced in males exposed to exogenous estrogens (Denslow et al. 1999). This abnormally high production of a female-specific protein in male fish has been used as a sensitive biochemical indicator of exposure to estrogenic chemicals in several fish species (Folmar et al. 1996, Harries et al. 1996, Porter and Janz 2003, Nakari 2004).

Mosquitofish (*Gambusia affinis*, Baird and Girard 1853) are sexually dimorphic fish. Males have an elongated anal fin, the gonopodium, which is used during reproduction. Elongation of the anal fin in developing males is under androgenic stimulation from the maturing testes (Turner 1941), and can be stimulated in juveniles of both sexes by exposure to androgens or inhibited by exposure to estrogens (Angus et al. 2001, Doyle and Lim 2002). This simple

indicator of exposure to estrogenic or androgenic stimulation, along with their restricted home-range, abundance, and near pan-global distribution (Bortone and Davis 1994, Overstreet et al. 1996, FishBase Internet) has made them a very popular species for diagnosing exposure to hormonally active chemicals in the environment (Batty and Lim 1999, Bortone and Cody 1999, Parks et al. 2001, Angus et al. 2002, Toft et al. 2003). Development of the gonopodium occurs in the first 40 to 60 days of life (Angus et al. 2001). Once fully developed, as indicated by the appearance of terminal hooks, it remains a permanent structure and does not grow or regress further under hormonal stimulation. This long duration and irreversibility make it an impractical biomarker for use in caging studies. Alternative short-term indicators of exposure to hormones need to be developed if mosquitofish are to be used in such studies.

A method to measure plasma Vtg in mosquitofish using an immunoblot assay has been recently developed (Tolar et al. 2001), but this technique requires drawing blood, a difficult procedure in such a small fish species (adult males are on average 2cm long). In this study, method development to measure liver Vtg mRNA induction in mosquitofish using RT-PCR is described.

## 7.3. Materials and Methods

### 7.3.1. Fish

Mosquitofish were captured from Lake Tarawera (Rotorua, New Zealand) with a beach seine net. Examination of the tip of the gonopodium of adult males confirmed this species as *G. affinis* (presence of internal spines on the distal end of ray 3; Rauchenberger 1989). Mosquitofish were transported back to the laboratory and kept in well-aerated 80-L glass aquaria in water collected from the Tarawera River supplemented with 2‰ NaCl as a disease preventative and to reduce osmotic stress, and maintained at approximately 25°C.

### 7.3.2. Exposure of mosquitofish to 17β-estradiol

Eighty adult male mosquitofish were chosen at random and allocated in groups of 20 to four aerated 10-L glass aquaria containing 8 L of Lake Tarawera water supplemented with 2‰

NaCl. Water temperature was kept constant at approximately 25°C. After a 1-d acclimatisation period, each aquarium received 50 µL of ethanol containing 17β-estradiol (E2; Sigma-Aldrich) at final concentrations of 0 (control), 1, 20, and 250 ng/L. Dissolved oxygen and temperature were measured daily with a YSI 55 meter (YSI Inc, Yellowsprings, OH, USA). Half the water in each aquarium was replaced and a new dose of E2 in 50 µL of ethanol added daily for 8 d. Fish were fed standard flake food (Nutrafin) every second day.

On day 4 and day 8, eight mosquitofish were removed from each of the four treatment tanks and anaesthetized with tricaine methanesulfonate (MS222, 0.1 g/L; Acros Organics, Belgium). Fish were killed by spinal severance and whole livers excised and stored in 200 µL of RNAlater® (Qiagen) in cryovials (Griener; Raylab, Auckland, New Zealand) at 4°C for 24 h, then at -80°C for long-term storage. All fish manipulations were done pursuant to the New Zealand Animal Welfare Act (1999).

### 7.3.3. RNA extractions

All work surfaces and tools were wiped with RNase AWAY® (Molecular BioProducts) immediately prior to extractions. Total RNA was extracted from five livers (selected at random from the eight available) from each treatment group (0, 1, 10, and 250 ng/L of E2) at each time point (4 and 8 d) with RNeasy® MinEluteTM spin columns (Qiagen) following the protocol for tissues described in the RNeasy Micro Handbook (Qiagen). Briefly, each liver was homogenized in buffer RLT (RNeasy Micro Kit, Qiagen) with three strokes of 10 s (IKA T8 Ultra-Turrax). The sample was cleaned by centrifugation, and the supernatant (containing RNA) combined with 70% ethanol and applied to an RNeasy MinElute column. The column was centrifuged, washed with buffer RW1 (RNeasy Micro Kit, Qiagen), centrifuged again, and then incubated for 15 min with a DNase I mix (Qiagen). After incubation, it was washed again with buffer RW1, dried with 80% ethanol, and RNA in the column matrix was eluted with RNase-free water. The eluate (12 µL) contained the extracted total RNA.

Total RNA was quantified using a RiboGreen® kit (RediPlateTM 96 RiboGreen RNA quantitation kit; Molecular Probes) as per the manufacturer's instructions. In short, serial dilutions of the samples and an RNA standard curve were incubated with RiboGreen reagent in a 96-well plate for 10 min at room temperature. After incubation, fluorescence was read with a fluorometer (FLUOStar model 403, BMG Lab Technologies) and compared with the

RNA standard curve to determine the amount of total RNA in each sample.

### 7.3.4. Primer design

Mosquitofish Vtg mRNA was sequenced by A. Laurie using the protocol described in Laurie (2004). Based on this sequence, LUX® primers were constructed using the LUX Designer software (Invitrogen). The primer sequences are presented in Table 7.1. The forward primer was labelled with a FAM fluorophore, while the reverse primer was unlabelled. The resulting Vtg mRNA amplicon had a design size of 92 bp.

**Table 7.1:** Primer sequences and melting temperatures (Tm) for vitellogenin mRNA and 18S rRNA in the western mosquitofish (*Gambusia affinis*). Lower-case letters indicate LUX-specific bases, required for the hairpin structure of the primer in the unbound form.

| Primer | Sequence | Tm (°C) |
|---|---|---|
| Fwd Vtg mRNA | cactgg AGG GAT GGT ATC CAA GAA CCA GtG * FAM | 64.5 |
| Rev Vtg mRNA | TTG CTC GCT ACG AAG ATT TGG A | 64.2 |
| Fwd 18S rRNA | catgc TGT GGG TGG TGG TGC AtG * JOE | 63.8 |
| Rev 18S rRNA | TGC CGG AGT CTC GTT CGT TA | 64.1 |

Preliminary studies indicated that the β-actin gene was not stably expressed in mosquitofish livers (data not shown), and 18S rRNA was therefore selected as the reference housekeeping gene. A 250-bp conserved region of the 18S rRNA sequence was identified with GenBank (Internet) sequences from several teleosts (*Oncorhynchus mykiss*, *Acanthopagrus latus*, *Chrysophrys major*, *Dentex dentex*, *Lateolabrax japonicus*, *Megalaspis cordyla*, *Pampus argenteus*, *Rastrelliger kanagurta*, *Siniperca chuatsi*, and *Trichiurus haumela*) using DNAMAN (Lynnon Corp, Vaudreuil, QC, Canada). Again, LUX primers were designed using the LUX Designer software (Table 7.1). The forward primer was labelled with a JOE fluorophore, and the reverse primer unlabelled. The resulting amplicon had a design size of 85 bp.

### 7.3.5. Reverse transcription and amplification of Vtg mRNA and 18S rRNA

Vtg mRNA and 18S rRNA were quantified in duplex using a one-step quantitative RT-PCR enzyme mix (SuperScript™ III Platinum® one-step quantitative RT-PCR system, Invitrogen) in an iCycler iQ real-time PCR detection system (Bio-Rad). The reactions were done in 96-well PCR plates (Bio-Rad) sealed with PCR sealing tape (Bio-Rad) to prevent evaporation of the 50-µL reaction mix (1 µL SuperScript III/Taq polymerase enzyme mix, 25 µL 2× reaction buffer, 1 µL RNase OUT, 1 µL Vtg forward primer [final concentration of 300nM], 1 µL Vtg reverse primer [600nM], 1 µL 18S forward primer [100nM], 1 µL 18S reverse primer [100nM], 9 µL RNase-free water, and 10 µL of sample diluted for a final concentration of 5 ng hepatic total RNA in each well) during the reaction. The thermal cycler was programmed for 1 cycle at 50°C for 20 min (RT step), 1 cycle at 95°C for 2 min for denaturation of the reverse transcriptase (SuperScript III) and activation of the polymerase (Taq), 45 cycles of 95°C for 15 s followed by 60°C for 30 s for the PCR reaction, concluded by melting curve analysis from 55°C to 91°C (180 cycles of 10 s, ramping of 0.2°C/cycle). The real-time fluorescence detector was set for JOE-530 (18S rRNA primers) and FAM-490 (Vtg mRNA primers).

Threshold cycles (Ct) for Vtg mRNA and 18S rRNA were estimated by analysis of the amplification curves with iQ 3.0a (Bio-Rad). ΔCt, a measure of the relative induction of Vtg mRNA, was calculated by subtracting the 18S rRNA Ct values from the Vtg mRNA Ct values.

### 7.3.6. Agarose gel electrophoresis

Several amplification products of the RT-PCR were run on agarose gel electrophoresis: *a)* Vtg mRNA primers and 5 ng of hepatic total RNA from an adult male exposed to 250 ng/L of estradiol (E2) for 8 d; *b)* Vtg mRNA primers and 5 ng of hepatic total RNA from a gravid female; *c)* Vtg mRNA primers and 5 ng of hepatic total RNA from an adult male exposed to 0 ng/L of E2 (control) for 8 d; and *d)* 18S rRNA primers and 5 ng of hepatic total RNA from an adult male. The RT-PCR products (10 µL) were loaded with 5 µL of loading dye and run for 1h at 100V in 3.5% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The gel was stained with ethidium bromide for 10 min, destained for 2 min, and a digital photograph of the gel taken with GelDoc 2000 (BioRad).

### 7.3.7. Statistical analysis

All statistical tests were undertaken with SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). The critical level of significance was set at $p = 0.05$ for all tests. A one-way analysis of variance (ANOVA) was used to test for differences in temperature and dissolved oxygen (DO) among the different exposure tanks. Differences in 18S rRNA Ct, Vtg mRNA Ct, and ΔCt values were tested separately for each exposure duration (4 and 8 d) with a one-way ANOVA followed by Bonferroni's test for multiple comparisons.

## 7.4. Results

### 7.4.1. Exposure conditions

The average temperature over the 8 d of exposure was 25.0 °C (ranging from 23.3 to 27.5°C). The average DO was 7.86 mg/L (ranging from 7.48 to 8.40 mg/L). There were no significant differences among any of the tanks in either temperature or DO.

### 7.4.2. Amplification products

RT-PCR with Vtg mRNA primers and RNA from an adult male exposed to 250 ng/L of E2 for 8 d produced a single amplicon with a melting temperature (Tm) of 80.6°C, a Ct of 25.9, and a size slightly less than 100 bp (Fig. 7.1, lane 2). With Vtg mRNA primers and RNA from a gravid female, an amplicon with the same Tm and size was obtained (Fig. 7.1, lane 3), but with a slightly lower Ct of 25.3. With Vtg mRNA primers and RNA from an adult male exposed to 0 ng/L of E2 for 8 d (control), an amplicon with a similar Tm of 80.8°C was obtained, but with a much higher Ct of 37.3 it was barely visible on the gel (Fig. 7.1, lane 5). Finally, with 18S rRNA primers and RNA from an adult male, a single amplicon with a melting temperature of 83.0°C, a Ct of 10.5, and a clear band at slightly more than 75 bp was produced (Fig. 7.1, lane 4).

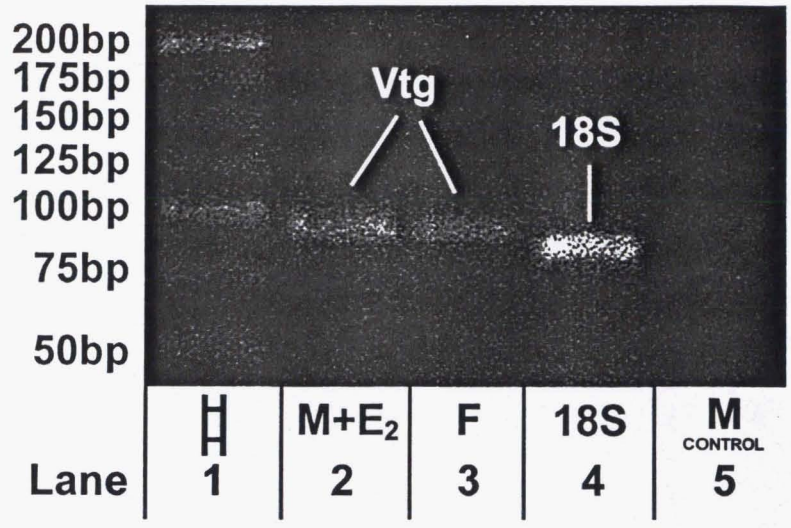


**Figure 7.1:** Agarose gel image of the reverse transcription-polymerase chain reaction (RT-PCR) amplification products in exposed and control mosquitofish (*Gambusia affinis*). Lane 1 is a DNA ladder. Lane 2 shows a single amplicon of approximately 90 bp after RT-PCR with vitellogenin (Vtg) primers and total RNA isolated from the liver of an adult male exposed to 250ng/L 17β-estradiol ($E_2$) for 8 days ($M+E_2$). Lane 3 also shows a single amplicon of approximately 90 bp after RT-PCR with Vtg primers and total RNA isolated from the liver of a gravid female (F). Lane 4 shows a single amplicon of approximately 80 bp after RT-PCR with 18S primers and total RNA isolated from the liver of an adult male. Lane 5 shows no amplification products after RT-PCR with Vtg primers and total RNA isolated from the liver of an adult male from the control group (after 8 days of exposure to 0ng/L $E_2$).

### 7.4.3. Ct and ΔCt values

There were no significant differences in 18S rRNA Ct values among any of the treatment groups after either 4 or 8 days of exposure (one-way ANOVA, $p = 0.201$ and $p = 0.763$, respectively; Table 7.2). Melting curve analysis revealed a single peak with a Tm of 82.9 ± 0.03°C (mode = 83.0°C).

There was a significant effect of $E_2$ treatment on Vtg mRNA Ct after both 4 and 8 days of exposure (one-way ANOVA, $p < 0.001$ in both cases). However, only mosquitofish exposed to the highest $E_2$ concentration (250ng/L) had significantly lower Ct values than the control group after both 4 and 8 days (Bonferroni's, $p < 0.001$ in both cases; Table 7.2). Melting curve analysis produced a single peak with a Tm of 80.4 ± 0.05°C (mode = 80.6°C).

**Table 7.2:** Vitellogenin (Vtg) mRNA and 18S rRNA expression in adult male mosquitofish after 4 and 8 days of exposure to 17β-estradiol ($E_2$). An asterisk indicates significant deviation from the respective control group (Bonferroni's, $p < 0.001$, $n = 5$). Vtg mRNA expression relative to the housekeeping gene 18S rRNA is calculated as ΔCt = (Vtg mRNA Ct) – (18S rRNA Ct). Vtg mRNA induction relative to the control group (0ng/L) is computed as $2^{-\Delta\Delta Ct}$, where ΔΔCt = (Treatment ΔCt) – (Control ΔCt).

| $E_2$ (ng/L) | 18S rRNA Ct ± SE | Vtg mRNA Ct ± SE | ΔCt ± SE | -ΔΔCt | Vtg mRNA induction |
|---|---|---|---|---|---|
| *Day 4* | | | | | |
| 0 (control) | 9.6 ± 0.3 | 38.6 ± 0.7 | 29.0 ± 0.6 | 0.0 | 1.0× |
| 1 | 10.2 ± 0.3 | 38.8 ± 0.5 | 28.5 ± 0.5 | 0.5 | 1.4× |
| 20 | 10.3 ± 0.3 | 38.9 ± 0.5 | 28.6 ± 0.4 | 0.5 | 1.4× |
| 250 | 9.8 ± 0.2 | 29.4 ± 1.4* | 19.6 ± 1.4* | 9.4 | 690× |
| *Day 8* | | | | | |
| 0 (control) | 10.3 ± 0.2 | 38.8 ± 0.9 | 28.5 ± 1.0 | 0.0 | 1.0× |
| 1 | 10.3 ± 0.2 | 39.5 ± 0.5 | 29.2 ± 0.5 | -0.7 | 0.6× |
| 20 | 10.3 ± 0.3 | 38.0 ± 0.9 | 27.6 ± 0.9 | 0.8 | 1.8× |
| 250 | 10.0 ± 0.3 | 25.4 ± 0.8* | 15.4 ± 0.7* | 13.1 | 8500× |

Consequently, there was a significant effect of $E_2$ exposure on relative Vtg mRNA expression (ΔCt) at both 4 and 8 days (one-way ANOVA, $p < 0.001$ in both cases), but only mosquitofish exposed to the highest concentration had a significantly higher Vtg mRNA expression relative to 18S rRNA expression (as indicated by the lower ΔCt value; Bonferroni's, $p < 0.001$; Table 7.2 and Fig. 7.2). This relative expression translated into a 690-fold induction of Vtg mRNA in mosquitofish exposed for 4 d to 250ng/L of $E_2$, and a 8500-fold induction after 8 d of the same exposure (Table 7.2).

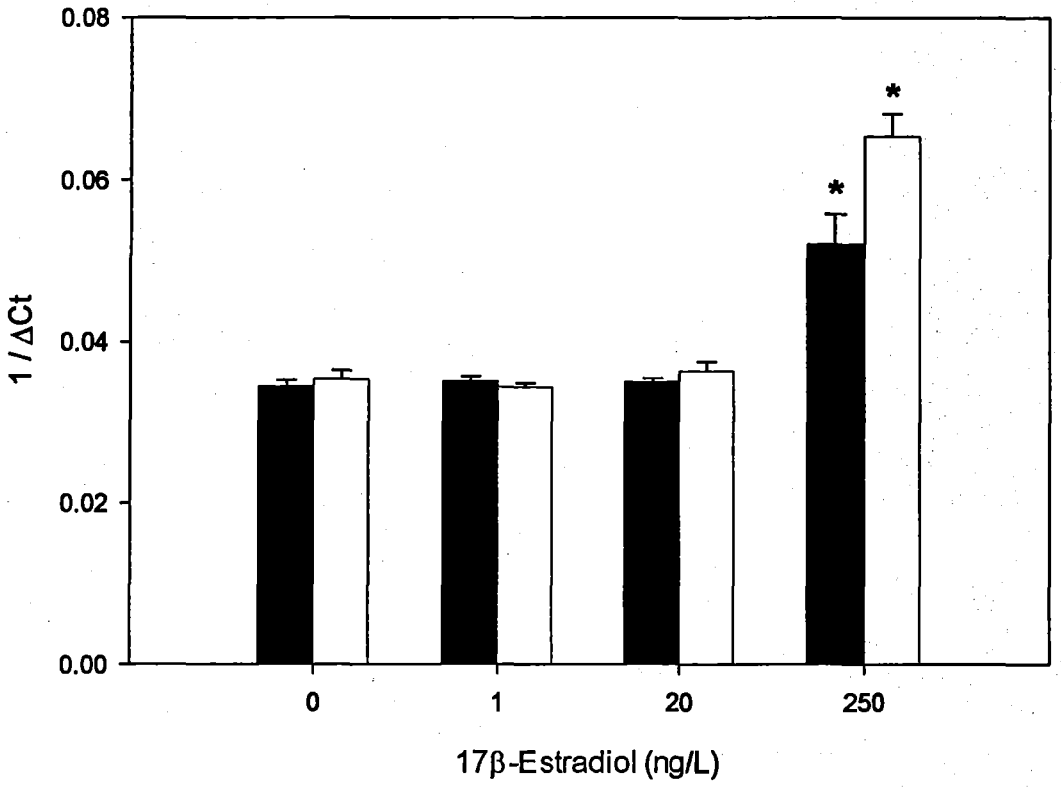


**Figure 7.2:** Induction of vitellogenin (Vtg) mRNA relative to the 18S rRNA housekeeping gene in adult male mosquitofish exposed to 0, 1, 20, and 250 ng/L of 17β-estradiol for 4 (■) and 8 d (□). Induction is expressed as 1/ΔCt, where ΔCt is the difference between the amplification threshold cycle (Ct) for Vtg mRNA and the Ct for 18S rRNA. The asterisk indicates statistically significance deviation from the control value (0 ng/L) at that sampling time.

## 7.5. Discussion

The RT-PCR method described in this paper allowed quantification of a significant Vtg mRNA induction in adult male mosquitofish after just 4 days of static waterborne exposure to 250 ng/L of $E_2$ (Fig. 7.2). This is similar to the results reported in Denslow et al. (2001), where male sheepshead minnows (*Cyprinidon variegatus*) exposed to 100 ng/L of $E_2$ in a flow-through system showed a significant induction of the Vtg mRNA after just 2 d. Due to their relatively quick disappearance from the water phase, the potency of the tested chemicals is often underestimated in static exposures. This may explain the apparent lower sensitivity of mosquitofish to E2 compared with sheepshead minnows. Most other studies on Vtg

mRNA induction have been done with injections of $E_2$ or waterborne exposure to the more potent, synthetic estrogen, ethinylestradiol ($EE_2$). Lattier et al. (2001) measured a significant Vtg mRNA induction in male carp (*Cyprinus carpio*) after 24 and 48h of an injection of 0.033 mg of $E_2$ per kilogram of body weight using an RT-PCR protocol similar to the one described in this study. Juvenile fathead minnows (*Pimephales promelas*) injected with a high dose of 5 mg of $E_2$ per kilogram of body weight showed a significant induction of Vtg mRNA within 24h (Thomas-Jones et al. 2003). A significant Vtg mRNA induction was detectable in mature male Japanese medaka (*Oryzias latipes*) exposed to 25 ng/L $EE_2$ for 7 d (Islinger et al. 2002). In this study, static exposure of adult male mosquitofish to 20 ng/L $E_2$ for 8 d did not significantly induce Vtg mRNA. However, $EE_2$ is more potent than $E_2$ *in vivo* (Islinger et al. 2002), and exposure to $EE_2$ was about twice as potent as the same dose of $E_2$ at inducing Vtg production in sheepshead minnows (Folmar et al. 2002). Therefore, it is not clear if mosquitofish are less sensitive than other species to induce Vtg mRNA, or if the differences are due to different exposure methods (static vs. flow-through) and/or chemical ($E_2$ vs. $EE_2$). A more complete gradient of $E_2$ exposure concentrations between 20 and 250 ng/L and exposure to $EE_2$ would help determine the sensitivity of mosquitofish relative to other species.

Real-time RT-PCR reactions are so sensitive to minute quantities of RNA that variations in RNA extraction efficiencies or minor pipetting inconsistencies can have a significant effect on the resulting Ct values (Bustin 2002). This problem was overcome in the present study by the use of an internal RNA standard. Ribosomal RNA has been suggested as a stable internal RNA normalizer (Zhong and Simmons 1999), and 18S rRNA was chosen as the housekeeping gene in this study. However, expression of 18S rRNA in mosquitofish liver was very high and the amount of total RNA added to each well had to be diluted to 5 ng per microplate well to obtain a sufficient baseline for accurate estimation of the threshold cycle (Ct). Unfortunately, this dilution also decreased the number of copies of the Vtg mRNA template and hence the sensitivity of the Vtg mRNA amplification. Use of a relatively less expressed housekeeping gene (such as β-actin) that would require less dilution of the samples may increase the sensitivity of this method. Nevertheless, expression of 18S rRNA was remarkably stable, with Ct values for all exposure groups around 10 cycles (Table 7.2) when 5 ng of total hepatic RNA was added to the reaction well. This stable expression suggests that it is a reliable housekeeping gene in *G. affinis* liver.

Compared with other assays to measure Vtg in mosquitofish, this RT-PCR method was sensitive and a significant induction was measurable after a very short period of time. No

other study has measured the effect of waterborne steroids on Vtg in mosquitofish, but an increase in plasma Vtg (measured by immunoblot assay) after 7 d of a dietary exposure was observed at 10 μg of ethinylestradiol ($EE_2$) per gram of food (Tolar et al. 2001). No data are available on the comparability of dietary and waterborne exposure to steroids in mosquitofish, so a "creative extrapolation" is necessary. Juvenile rainbow trout exposed for 7 d to 100 ng/L $EE_2$ had about half the plasma Vtg levels of juvenile rainbow trout exposed to 10 μg $EE_2$ per gram of food for the same time period (Verslycke et al. 2002). Assuming a similar relationship (ie 10 μg/g food is equivalent to 200 ng/L) for mosquitofish, a rough estimate of the equivalent waterborne threshold for the immunoblot assay after 7 d of exposure would be approximately 200 ng/L $EE_2$, or the equivalent of 400 ng/L $E_2$. This value is close to the 250 ng/L used in this study. From a practical point of view however, the RT-PCR method is easier to carry out than the immunoblot method, as extraction of whole livers from mosquitofish carcasses is relatively simple and could potentially be carried out in the field (with RNA adequately preserved in RNAlater solution).

Gonopodium development in mosquitofish juvenile males was significantly affected after 84d of exposure to 100 ng/L $E_2$ (Doyle and Lim 2002). A significant estrogenic effect could be measured within 4 d of exposure to a slightly higher concentration (250 ng/L $E_2$) with the RT-PCR method described in this paper. The shorter time period required before a significant effect was measurable means that the RT-PCR method could be used in short-term field-caging studies to measure estrogenic induction at the caging site. It may also be used to determine if wild mosquitofish populations are being exposed to estrogenic chemicals, although the effect of long-term exposure to estrogenic stimulation on Vtg mRNA is not known. Studies with male sheepshead minnows suggest that Vtg mRNA levels remain high even after 3 weeks of a continued waterborne exposure to low levels of estradiol (100 ng/L $E_2$) (Denslow et al. 2001). This suggests that male mosquitofish chronically exposed to estrogenic chemicals would continue to have higher levels of Vtg mRNA than reference male fish.

More research is needed to link the increase in Vtg mRNA levels in male mosquitofish with increased Vtg synthesis, and to determine the usefulness of the method in field monitoring.

## 7.6. Co-author contributions

This chapter has been submitted to Biomarkers. Besides supervisory committee members, co-authors for this paper are Mike van den Heuvel (Forest Research, Rotorua, New Zealand) and Andrew Laurie (Canterbury Health Laboratories, Christchurch, New Zealand). Mike van den Heuvel helped with sampling of mosquitofish and provided logistical support during exposures, which were carried out at the Forest Research fish facilities. Andrew Laurie provided the gene sequence for mosquitofish Vtg mRNA, and the Vtg mRNA primers designed in this study are based on that sequence.

# 8. General discussion

## 8.1. General discussion

The levels of estrogenic and androgenic activity in raw sewage in this study were similar to those reported in Europe and Japan (Kirk et al. 2002, Murk et al. 2002, Onda et al. 2002), from <4 to 185 ng/L EEq and 1920 to 9330 ng/L TEq (Chapter 5). In this study, the samples were taken from STPs in urban areas such as Brisbane and Christchurch, which have population densities very similar to some European cities. However, the lower overall population density in Australia and New Zealand compared to Europe or Japan (2.6 and 15 people/km$^2$ in Australia and New Zealand compared to 246 and 337 people/km$^2$ in the United Kingdom and Japan, respectively; CIA 2004) is likely to result in lower concentrations of EDCs in the receiving environment. More importantly, many cities in Australia and New Zealand are located in coastal areas. Treated sewage is therefore often discharged directly into the ocean, where dispersion and dilution of sewage occur more rapidly than in rivers. The combination of these factors suggests that the areas impacted by treated sewage discharges would be relatively small and the effect on wildlife likely less. This issue has to be taken into account when attempting to characterize the risks associated with EDCs in treated sewage.

The treatment plants in Australia and New Zealand that were tested in this study were very effective at removing most of the estrogenic and androgenic activity in sewage, with levels in the final effluents between <1 and 4.2 ng/L EEq and <6.5 to 736 ng/L TEq (Chapter 5). Two *in vitro* bioassays were used in this study to provide an estimate of the estrogenic and androgenic activity of municipal sewage: the sheep ER and the rainbow trout AR binding assays. The sheep ER binding assay yielded very similar results to those reported for a rat ER binding assay advocated by the US Environmental Protection Agency as an appropriate Tier I screening assay (EDSTAC 1998, ICCVAM 2003) (Chapter 3). The assay can also be used to evaluate the estrogenic potency of individual compounds (Chapter 3). The ER binding assay is best used in a tiered approach to screen for estrogenicity, and proved to be a very effective screening tools to evaluate cumulative estrogenic activity in sewage extracts. One of the limitations of receptor binding assays however is that only chemicals that affect endocrine systems via receptor-mediated pathways would elicit a significant response. Chemicals that interfere with other endocrine pathways, such as hormone metabolism, would not be identified as active in a receptor binding assay. In wastewater with a predominantly domestic

source, the estrogenic activity is most likely due to natural and synthetic hormones excreted by humans (Desbrow et al. 1998). The mechanism of action of these steroid hormones *in vivo* involves interaction with the ER. Likewise, most of the androgenic activity in domestic sewage is most likely caused by natural androgens, which would naturally bind to the AR. Receptor binding assays would therefore be appropriate to test hormonal activity in this type of wastewater. Results from the ER binding assay were positively correlated with those of an E-Screen (Chapter 3), suggesting that ER binding was a good indicator of the full cellular response. Actual field data comparing the two assays (Chapter 4) illustrated differences between the two assays, with estrogenicity measured with the E-Screen generally lower than with the ER binding assay. This is attributable to the difference in biological complexity between the two assays. The E-Screen is a cellular endpoint, and integrates the full response of cells to estrogenic compounds. This includes crossing of the cell membrane, binding to the ER, activation of the ER, as well as the whole genetic machinery involved in transduction, transcription, and translation of the signal. The ER binding assay on the other hand is a molecular endpoint, and estrogenicity is measured solely by the ability of chemicals to displace the native ligand from the ER binding site. The ER binding assay can thus be thought of as a bioassay for potential receptor-mediated estrogenic disruption, while the E-Screen is more representative of the full cellular response. It is comforting to see that even with the general overestimation of estrogenicity inherent to binding assays (Kinnberg 2003), the activity of the final effluents at all STPs sampled was below 4.2 ng/L EEq, indicative of very low estrogenicity. It is important, however, to realize that these *in vitro* bioassays do not account for the complex feedback mechanisms that occur *in vivo* and the results must be interpreted with caution. For instance, when comparing $E_2$ equivalency factors (EEF, a measure of relative potency) determined from an ER binding assay with those determined *in vivo* with transgenic zebrafish, Legler et al. (2002) showed that some synthetic chemicals were more potent *in vivo* than in the binding assay. The results of the *in vitro* assays in this study were however confirmed by the lack of significant effects in mosquitofish exposed to fully-treated sewage (Chapter 6).

Mosquitofish sampled at a site receiving undiluted secondary-sewage effluent with the highest levels of estrogenic and androgenic activity (site A1 received effluent from plant F1A, which had 6.4 ng/L EEq and 2290 ng/L TEq, respectively) had slightly elongated anal fins compared with mosquitofish sampled further downstream or at a reference site (Chapter 6). This androgenic effect did not however appear to have a detrimental effect at the population level,

as indicated by the large number of mosquitofish that were captured at that site. The levels of androgenic and estrogenic activity in all final effluents were well below these concentrations (Chapter 5), suggesting that exposure to treated sewage from these plants is unlikely to cause reproductive abnormalities in exposed mosquitofish populations. It is not known how sensitive the mosquitofish biomarkers are in comparison to responses in other aquatic species. There is very little information on the effects of EDCs on Australian and New Zealand native species, and more research is needed to determine if the mosquitofish endpoints are adequate to ensure other potentially more sensitive species are also protected. The RT-PCR method to measure vitellogenin mRNA in mosquitofish (Chapter 7) should provide a more sensitive *in vivo* biomarker that could be used to quickly estimate estrogenic activity in the field and in laboratory exposures. The assay needs to be further validated in the field and against biochemical parameters, to establish the link between copies of mRNA and protein expression. Caging studies could then be used systematically to provide an estimate of the estrogenic activity of point sources, such as industrial and sewage treatment outfalls, which may help to prioritize environmental research initiatives and identify industries or geographical areas for intensive monitoring of EDCs.

## 8.2. Conclusions

In conclusion, the estrogenic and androgenic activity of treated sewage in Australia and New Zealand is very low, as indicated by *in vitro* molecular and cellular bioassays as well as *in situ* sampling of mosquitofish. This suggests that the potential for significant endocrine disruption associated with treated municipal sewage discharges in Australia and New Zealand is minimal, as both the dose and the exposure areas are very small. However, more research is needed to determine the hormonal activity in industrial and agricultural wastewater, as well as the sensitivity of endemic Australian and New Zealand species to endocrine disruption.

With regards to the initial hypotheses:

- EDCs are present in Australian and New Zealand sewage, as indicated by the high estrogenicity and androgenicity of raw sewage (Chapter 4 and 5) and by chemical analysis (Chapter 4).

- Of all treatment technologies examined, activated sludge was the most effective at removing the estrogenic and androgenic activity in sewage (Chapter 4 and 5).

- Although mosquitofish exposed to incompletely-treated sewage had elongated anal fins (consistent with exposure to androgenic chemicals), mosquitofish exposed to fully-treated sewage did not exhibit morphological signs of endocrine disruption (Chapter 6).

- Exposure of adult male mosquitofish to estrogens significantly induced Vtg mRNA expression after just 4 d of exposure, but only at the highest exposure concentration (250 ng/L $E_2$; Chapter 7).

## 8.3. Future work

The focus of this study was on hormonal activity associated with municipal sewage with mostly domestic inputs. The bioassays developed during this project (Chapter 3 and 7) could however be used to test the estrogenic or androgenic activity of other types of wastewater. Industrial chemicals such as phthalates, alkyphenols, and bisphenol A have a significant estrogenic activity (Johnson and Jürgens 2003), and are present in high concentrations in wastewater from different types of industries (Lee et al. 2002). Although these industrial xenoestrogens are not as potent as natural hormones, they are not as readily degraded and are therefore more persistent in the environment. Agricultural wastes are another type of wastewater of particular concern, particularly in Australia and New Zealand where agriculture is a major sector of the economy. Recently, the ER binding assay was used successfully to test the estrogenicity of agricultural waste (Sarmah et al. 2005). Livestock excrete much higher levels of natural hormones than humans (Shore and Shemesh 2003), and agricultural waste (such as dairy farm effluent) is only marginally treated before discharge into the environment. An assessment of the hormonal activity in treated agricultural and industrial wastewater should be carried out to determine their potential for endocrine disruption.

Other studies have indicated that lipophilic chemicals may associate with sludge during treatment in activated sludge reactors (Layton et al. 2000, Esperanza et al. 2004), and estrogenic and androgenic activity in the sludge can therefore be relatively high (Murk et al. 2002). Other synthetic chemicals such pharmaceuticals and personal care products (PPCPs) are also present in high concentrations in domestic sewage (Ingerslev et al. 2003) and most likely also segregate into sludge. It is however unclear how long estrogenic and androgenic chemicals survive in the sludge. The total biological activity in sludge from STPs should be

assessed, particularly considering that sludge is increasingly applied to land as fertilizer.

Once the Vtg mRNA RT-PCR method has been fully validated, caging studies with mosquitofish could be undertaken to identify point sources of EDCs in the aquatic environment. Mosquitofish are tolerant of a wide range of ecological conditions (Overstreet et al. 1996), and could be caged in a wide range of riverine and brackish water environments to determine environmental health.

Finally, more studies need to be done with endemic species to address the lack of information to properly characterize the risk of endocrine disruption to the unique Australian and New Zealand fauna.

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