ASSESSING THE ROLES OF ANTI-ANDROGENIC AND OESTROGENIC MIXTURES ON ENDOCRINE DISRUPTION IN FISH

A thesis submitted for the degree of

Doctor of Philosophy

by

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DECLARATION

The work submitted in this thesis was conducted between 2010 and 2013 at Brunel University's Institute for the Environment (Uxbridge, UK). This work was carried out independently and has not been submitted for any other degree.

STATEMENT OF CONTRIBUTION

In chapter two, I completed all desk based modelling of sewage effluent concentrations of oestrogens and anti-androgens and ran the Source Catchments model. In chapters two and three, data from the Low Flows 2000-WQX model were produced by Richard Williams of the Centre for Ecology and Hydrology, Wallingford. I conducted the *in vivo* experiments under the guidance of Dr Jayne Brian of Brunel University. I also completed both histology and ELISAs, whilst statistical analysis for experiment two was completed by Martin Scholze of Brunel University. I also conducted the analytical chemistry under the guidance of Professor Rakesh Kanda, at the time of Severn Trent Services, Reading. In chapter four, sample preparation, fractionation and GC-MS were conducted by members of Severn Trent Services, Reading. I completed the literature searches from the GC-MS output and determined appropriate compounds for screening. Yeast Screens were conducted at Brunel University by Dr Alice Baynes and Nicola Beresford and I completed the data interpretation.

ABSTRACT

Incidence of endocrine disruption in wild fish species has been documented globally and is well characterised in the UK, where the occurrence of intersex in roach (*Rutilus rutilus*) is widespread. Although this has been associated with concentrations of steroid oestrogens, research indicates that anti-androgenic chemicals may also play a role in inducing these effects. Anti-androgenic activity is commonly detected in wastewater treatment works effluents and some receiving waters, but the chemicals responsible remain largely uncharacterised. This thesis aimed to identify environmental anti-androgens in UK and South Australian catchments and to produce environmentally relevant exposures to assess their potential impacts on sexual disruption in fish, alone and in combination with steroid oestrogens. By using hydrological modelling techniques, pharmaceuticals with an anti-androgenic mode of action were predicted to occur in the ng/L concentration range in UK and South Australian wastewater treatment works effluents and river catchments. This work included analysis of future trends in environmental concentrations of the pharmaceuticals and the steroid oestrogens in these catchments. Modest increases in concentrations by 2050 were predicted in the absence of mitigation, which could increase in the risk posed to fish health by the steroid oestrogens in the future. The effects of the predicted concentrations of two pharmaceuticals, bicalutamide and cyproterone acetate, were then assessed in fathead minnows (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*) based on the UK modelling for the present day. These concentrations did not contribute to endpoints characteristic of sexual disruption, alone or in combination with steroid oestrogens. However, the results did support an environmental role for the steroid oestrogens in intersex induction. Concurrently, effect directed analysis identified some highly potent anti-androgens, such as triclosan and pyrene, in wastewater treatment works effluents from the UK. However, they are likely to make a minor contribution to overall anti-androgenic activity due to their low concentrations. Consequently, more work is required to identify the causes of this activity in the environment and its implications for wild fish health.

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Time seems to have gone by incredibly fast since I was first lucky enough to join Brunel's Institute for the Environment and I now find myself finally at the end of my thesis and looking retrospectively at my experiences over the last four years. Firstly, I would like to extend a general thanks to the members of IFE. It has been both a pleasure and a privilege to work with such a dedicated and passionate group of people. Your input, advice and motivation has been a constant support to me throughout my PhD and I wish you all the best of luck in the future.

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PUBLICATIONS

Research published in peer reviewed journals and presented at conferences which originated from this thesis is shown below:

- **Green, C.**, Brian, J., Kanda, R., Scholze, M., Williams, R. and Jobling, S. Environmental concentrations of anti-androgenic pharmaceuticals do not impact sexual disruption in fish alone or in combination with steroid oestrogens. **2014** *Aquatic Toxicology.* In Press.
- **Green, C.**, Williams, R., Kanda, R., Churchley, J., He, Y., Thomas, S., Goonan, P., Kumar, A. and Jobling S. Modelling of steroid estrogen contamination in UK and South Australian rivers predicts modest increases in concentrations in the future*. Environmental Science and Technology.* **2013**, 47 (13), 7224–7232. DOI: 10.1021/es3051058.
- **Green, C.**, Brian, J., Williams, R., Scholze, M. and Jobling, S. The Effects of Anti-Androgenic Pharmaceuticals on Oestrogen Induced Feminisation in Model Fish Species. *Platform presentation. 23rd SETAC European Meeting, Glasgow, 2013.*
- **Green, C.**, Williams, R., Kanda, R., Churchley, J., He, Y., Thomas, S., Goonan, P., Kumar, A. and Jobling S. Predictive modelling of steroid oestrogens in sewage effluent demonstrates the potential for endocrine disruptive effects in wild fish populations in South Australia. *Poster Presentation. 6th SETAC World Congress, Berlin, 2012.*
- **Green, C.**, Baynes, A., Kanda, R., Churchley, J. and Jobling, S. Wastewater analysis may provide clues to the chemical causes of endocrine disruption. *Poster Presentation. SETAC UK Branch Annual Meeting 2011.*

Additional research published but not included in this thesis:

- Baynes, A.; **Green, C.**; Nicol, E.; Beresford, N.; Kanda, R.; Henshaw, A.; Churchley, J.; Jobling, S. Additional Treatment of Wastewater Reduces Endocrine Disruption in Wild Fish: A Comparative Study of Tertiary and Advanced Treatments. *Environmental Science and Technology.* **2012***,* 46 (10)*,* 5565-5573. DOI: 10.1021/es204590d

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DEFINITIONS

ABS	Australian Bureau of Statistics
ADHD	Attention Deficit/Hyperactivity Disorder
AhR	Aryl Hydrocarbon Receptor
ANOVA	One Way Analysis of Variance
AP-1	Activator protein 1
AR	Androgen Receptor
ASP	Activated Sludge Process
BPA	Bisphenol A
COX	Cyclooxygenase
CPRG	Chlorophenol Red β-Galactopyranoside
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DBP	Di Butyl Phthalate
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di (2-ethylhexyl) Phthalate
DES	Diethylstilbestrol
DHT	5α-Dihydrotestosterone
DWF	Dry Weather Flow
E1	Oestrone
E2	17β-Oestradiol
EDA	Effect Directed Analysis
EDC	Endocrine Disrupting Chemical
EE2	17α-Ethinylestradiol
EEQ	17β-oestradiol equivalent
ELISA	Enzyme Linked Immunosorbant Assay
EQS	Environmental Quality Standards
EU	European Union
FluEQ	Flutamide Equivalent Concentration
FSH	Follicle Stimulating Hormone
GAC	Granular Activated Carbon
GC-MS	Gas Chromatography-Mass Spectrometry
GFP	Green Fluorescent Protein
GnRH	Gonadotrophin Releasing Hormone
GSI	Gonadosomatic Index
HHCB	Galaxolide
HPG axis	Hypothalamic-Pituitary-Gonadal Axis
HPLC	High Performance Liquid Chromatography
HRT	Hormone Replacement Therapy
HSI	Hepatosomatic Index
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LF2000-WQX	The Low Flows 2000 Water Quality eXtension model
LH	Luteinising Hormone
LOD	Limit of Detection
LOEC	Lowest Observed Effect Concentration
MFS	Modified FETAX (Frog Embryo Teratogenesis Assay-Xenopus) Solution
MRM	Multiple Reaction Monitoring
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NOEC	No Observed Effect Concentration
NP	4-Nonylphenol
NSAID	Non-Steroidal Anti-Inflammatory Inhibitors
ONS	Office for National Statistics

OP	Octylphenol
PAH	Poly Aromatic Hydrocarbons
PBDE	Polybrominated Diphenyl Ethers
PCB	Polychlorinated Biphenyls
PFOA	Perfluorooctanoic acid
PNEC	Predicted No Effect Concentration
POP	Persistent Organic Pollutants
PPAR	Peroxisome Proliferating Activated Receptor
REACH	Registration, Evaluation, Authorisation & Restriction of Chemicals
SPE	Solid Phase Extraction
TIE	Toxicity Identification Evaluation
UNEP	United Nations Environmental Program
WWTW	Wastewater treatment Works
YAS	Yeast Androgen Screen
YES	Yeast Oestrogen Screen

CHAPTER 1: GENERAL INTRODUCTION

1.1 Background

Between 2000 and 2009, annual global sales of products from the chemical industry doubled and projections anticipate a 3% annual rise up to 2050. The exact number of chemicals on the global market is unknown, although 143,835 have been preregistered for the European Union (EU) regulation REACH (Registration, Evaluation, Authorisation & Restriction of Chemicals) and this is likely to be an underestimation of the total. These chemicals are diverse in their uses and include basic/commodity chemicals (such as petrochemicals and inorganic chemicals), speciality chemicals derived from basic compounds (such as plasticisers and catalysts), pesticides, pharmaceuticals and personal care products (Sigman *et al.*, 2012). Some of these chemicals are beneficial to human health and wellbeing and they all benefit the economy. However, many have been shown to have negative impacts on human and environmental health as emission from industry, agriculture and human waste has made anthropogenic chemical contamination of the environment ubiquitous.

In the past, awareness of the negative impacts of anthropogenic pollution was characterised by direct effects on human health. In particular, there was a focus on localised events which caused significant mortality, such as the Great Smog in London in 1952, which caused 4,000 deaths during its five day course and provided the impetus for legislation in the form of the Clean Air Act in 1956 (Hoffmann, 1993). The same can be said for awareness of the effects of pollution on wildlife, which focussed on localised poisoning events, such as maritime oil spills, mining discharges and lead toxicity linked to the death of birds (Rattner, 2009). However, the publication of Silent Spring by Rachel Carson in 1962 brought the issue of pollution and wildlife health to the forefront of public and political attention. This broadened the scope for chemical effects by linking pollutants, such as the organochlorine insecticide dichlorodiphenyltrichloroethane (DDT), not just to death but also to reproductive failures in non-target organisms. It highlighted the ability of these compounds to persist and bioaccumulate in food webs, leading to dramatic population declines in bird species (Carson, 1962). In particular, it considered that pollutants could have effects on entire ecosystems and that the human body could also become contaminated. Indeed, even in countries where bans have been implemented for decades, a legacy of the use of DDT remains as it is still detected in breast milk, (Shen *et al.*, 2007).

It is only in the last few decades that the ability of some chemicals to interfere with the endocrine system in humans and animals has been recognised. These chemicals are termed “endocrine disruptors” and are defined as: *“an exogenous substance or mixture*

that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations. A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations" (International Programme on Chemical Safety, 2002). Endocrine disrupting chemicals (EDCs) consist of a diverse range of over 800 chemicals from different classes which include, but are not limited to, pesticides, plasticisers, pharmaceuticals and personal care products, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, flame retardants, natural hormones and heavy metals. These can enter the environment during their production, use and disposal, at point sources such as wastewater treatment works (WWTWs) (e.g. pharmaceuticals) or through runoff from urban or agricultural settings (e.g. pesticides). Others can leach from a product during its lifespan (e.g. plasticisers) or they can be released from the environment by human activity (e.g. heavy metal contamination from mining). Many persist for long periods after their release, whilst others are constantly released into the environment, making them "pseudopersistent". Furthermore, some EDCs can be transported long distances through natural processes, whilst others are in use around the world, leading to global exposure of humans and wildlife to these pollutants (WHO/UNEP, 2013).

In response to the realisation that a variety of chemicals could exert effects through endocrine disruption, some countries have set up frameworks to assess suspected endocrine disruptors. These include the chemicals regulation REACH in the EU, the Endocrine Disruptor Screening Program run by the US Environmental Protection Agency and the Japanese Ministry for the Environment's Extend 2005 and 2010 programs. The science behind endocrine disruption and EDCs has also recently been assessed in a number of reports from the World Health Organisation and the United Nations Environmental Program (UNEP) (State of the Science of Endocrine Disrupting Chemicals – 2012), the European Commission (State of the Art Assessment of Endocrine Disrupters, 2011) and the European Environment Agency (The Impacts of Endocrine Disrupters on Wildlife, People and Their Environments: the Weybridge+15 [1996–2011] Report). These conclude that EDCs are capable of affecting human and wildlife health and constitute a global threat. However, continued research is required to advance our understanding of these pollutants. Indeed, expanding the identification of potential EDCs, characterising their exposure to humans and wildlife and determining how and when they act both singularly and in combination with other EDCs, is increasingly important. What is more, there have been calls from scientists for

regulatory action to mediate their potential risks and to avoid irreversible harm in the future in the 2013 Berlaymont Declaration.

1.2 Example classes of endocrine disruptors

1.2.1 Persistent and bioaccumulative halogenated chemicals

The Stockholm Convention was set up by UNEP in 2001 to reduce and eventually eliminate the global use of persistent organic pollutants (POPs). This initially included 12 chemicals but this has since been updated to 22. These include pesticides, such as DDT, dieldrin and endosulfan, as well as industrial chemicals and byproducts, such as the polychlorinated biphenyls (PCBs), which are complex mixtures used primarily as insulating agents, and polybrominated diphenyl ethers (PBDEs), which are used as flame retardants. All are highly persistent in the environment and bioaccumulate in food chains, which has led to them being identified in animals at higher trophic levels and humans globally, even following bans on their use (Shen *et al.*, 2007). They are also subject to long range atmospheric transport, which has led to their detection in "pristine" areas far from their origin. Indeed, these contaminants have been detected in the Arctic and its wildlife, as well as the people who depend on these animals as a food source, demonstrating the global relevance of the issue (Fisk *et al.*, 2005; Van Oostdam *et al.*, 2005). In addition to the initial POPs, other persistent and bioaccumulative chemicals are now under review for inclusion in the Stockholm Convention. These include perfluorooctanoic acid (PFOA) and brominated flame retardants, for which evidence of endocrine effects are increasing and their detection in the human body has occurred globally (White *et al.*, 2011; Watanabe and Sakai, 2003).

1.2.2 Less persistent and less bioaccumulative chemicals

Whilst these chemicals are easier to break down than POPs and do not have the same potential to bioaccumulate, their use and release into the environment is widespread and constant. As a result, they have been termed "pseudo-persistent." For example, plasticisers including the phthalate esters, di(2-ethylhexyl)phthalate (DEHP) and dibutylphthalate (DBP), as well as bisphenol A (BPA), are some of the most abundant anthropogenic chemicals in the environment (Oehlmann *et al.*, 2009; Murature *et al.*, 1987). They are used in manufacturing to increase flexibility in polymers and are found in many everyday objects including food wrapping, pesticides and medical equipment (Lovekamp-Swan and Davis, 2003). However, they are not covalently bound to the

product and can leach out into the environment. Alkylphenolic chemicals, such as nonylphenol (NP), octylphenol (OP) and their ethoxylates, also have an industrial usage as non-ionic surfactants and enter the aquatic environment through industrial discharge. Although they have now had their use restricted in the EU, due to concerns for their acute toxicity, they are still detected at lower concentrations (Soares *et al.*, 2008). Similarly, some personal care products enter the environment through disposal routes from households, such as the biocide triclosan, which originates from soaps, skin creams and toothpastes (von der Ohe *et al.*, 2012). In contrast, polycyclic aromatic hydrocarbons, such as benzo[a]pyrene and anthracene, originate from industrial processes as byproducts of the incomplete combustion of fossil fuels, including vehicle emissions (Kizu *et al.*, 2003b; Vinggaard *et al.*, 2000). Although they are combustion products, they are not only air pollutants and can be detected in other environmental media, particularly following their discharge in industrial effluents or urban runoff (Eisler, 1987).

1.2.3 Current use pesticides and pharmaceuticals

Both pesticides and pharmaceuticals are designed to be biologically active and have become contaminants of emerging concern due to their potential effects on non-target organisms and their constant release into the environment. Whilst current use pesticides do not have the same persistence or ability to bioaccumulate as their predecessors, many still have endocrine activity and experience extensive use in agriculture as well as in building material and garden care (WHO/UNEP, 2013). Indeed, almost 60 pesticides are now listed for testing in the US Environmental Protection Agency's Endocrine Disruptor Screening Program, many of which are in current use in the USA, such as atrazine and vinclozolin. However, this is only a proportion of the plethora of pesticides with known endocrine activity. Indeed, one study tested over 200 pesticides *in vitro*, including many in current use, and found 80 with oestrogenic activity, 66 with anti-androgenic activity and 34 with combined activity (Kojima *et al.*, 2004). Pharmaceuticals are of particular relevance to the aquatic environment since they are discharged constantly through wastewater treatment works effluent following human excretion. Improper disposal and manufacturing can also have an impact and there are some exceptional cases where effluents from manufacturing plants in third world countries contained very high concentrations of antibiotics (Larsson *et al.*, 2007). Consequently, a plethora of drugs have now been detected worldwide and include beta blockers, antibiotics, progestagens, non-steroidal anti-inflammatory drugs and selective serotonin reuptake inhibitors (reviewed by Corcoran *et al.*, 2010). One of the most

notable is 17α-ethinylestradiol (EE2), the active oestrogen in the contraceptive pill hormone, which will be subject to further discussion for its implication in the sexual disruption of wild fish.

1.2.4 Natural hormones, phytoestrogens and heavy metals

Natural hormones are highly active chemicals as they are natural ligands for hormone receptors within the endocrine system. In a majority of cases, this makes them more potent than other EDCs that mimic hormone action (Metcalfe *et al.*, 2001). The exceptions are some pharmaceuticals, which are designed to interact with receptors and to resist metabolic degradation, such as EE2 (Caldwell *et al.*, 2012). Natural hormones including testosterone, 17β-oestradiol (E2) and oestrone (E1) are all excreted by humans and animals. As a result, they constantly enter the aquatic environment via wastewater treatment works effluent and agricultural runoff from livestock farming and can also be described as pseudo-persistent (Matthiessen *et al.*, 2006; Johnson and Williams, 2004). Similarly, phytoestrogens derive naturally from plants and include isoflavonoids, such as genistein, and coumestans, such as coumestrol. Exposure of humans to these chemicals can occur through the diet, from foods including nuts and oils, soy products, cereals and breads (Thompson *et al.*, 2006). Exposure and effects in livestock has also been demonstrated through grazing, which can lead to infertility (Adams, 1995). Heavy metals also have a natural origin and are released from rocks through weathering processes. Their environmental presence is also arguably one of the oldest forms of anthropogenic pollution following the discovery of mining techniques. Indeed, environmental contamination increased dramatically following the industrial revolution, as the demand for metals rose along with their release through industrial processes (Nriagu, 1996). The use of heavy metals has since broadened, with cadmium used in batteries, arsenic used as a pesticide and lead incorporated into paints and fuels, producing a variety of exposure pathways for both humans and wildlife (WHO/UNEP, 2013).

1.3 The endocrine system

To understand the global concern surrounding EDCs, it is necessary to understand the function of the endocrine system. This has been well characterised in the WHO/UNEP report (WHO/UNEP, 2013), which states that human and wildlife health depends on the ability to reproduce and develop normally, and that this is not possible without a healthy

endocrine system. The endocrine system is a chemical messaging system made up of networks of ductless glands, organs, tissues and cells, which secrete hormones directly into the blood to regulate various bodily functions. This includes specialised endocrine glands, such as the thyroid or pituitary, as well as organs with secondary endocrine functions, such as the heart and liver (shown in Figure 1.1 below). Hormones are molecules derived from amino acids, cholesterol or phospholipids, which are produced by endocrine glands and travel via the blood to produce effects on distant target organs, tissues and cells (Melmed *et al.*, 2011). Whilst this can be described as endocrine action, hormones can also act on their parent cells (autocrine action), adjacent cells (paracrine action) and the nervous system (neuroendocrine action). Their circulating concentrations and actions within the body are controlled by complex feedback loops which determine their rate of production and secretion, as well as their metabolism and excretion.

Hormones have multiple functions within the human body in the control of physiological processes. Some are critical to the normal development of an organism and cause irreversible changes to morphology. For example, during male development in mammals, a bipotential gonad differentiates into the testes based on the presence of the Y encoded gene *sry*, without which an ovary forms. Secondary development of the reproductive tract, including the development of the male duct system and external genitalia is then driven by the testicular hormones, testosterone, anti-müllerian hormone and insulin-like peptide 3 (*insl3*) (Wilhelm *et al.*, 2007; Nef and Parada, 2000). Aspects of post-natal development, such as secondary sexual characteristics, are also linked to sex hormones controlled by the hypothalamic-pituitary-gonad axis, which also plays an important role in the reproductive cycle in later life (Ankley and Johnson, 2004). The endocrine system also maintains the internal environment through the feedback loops of hormones. For example, insulin plays a key role in controlling the metabolism and storage of glucose. It is secreted from the pancreas in response to increased plasma glucose concentrations, inhibits glucose production in the liver and promotes its uptake, utilisation and conversion to glycogen for storage in fat and muscle tissue. The reduction in blood glucose then negatively feeds back on the pancreas to reduce insulin secretion (Saltiel and Kahn, 2001). This regulation of the internal environment also allows an organism to adapt to changes in the external environment. For example, under stressful situations the glucocorticoid cortisol is released, which mobilises energy reserves by promoting gluconeogenesis and lipolysis to overcome the increased metabolic demand (Kudielka and Kirschbaum, 2005).

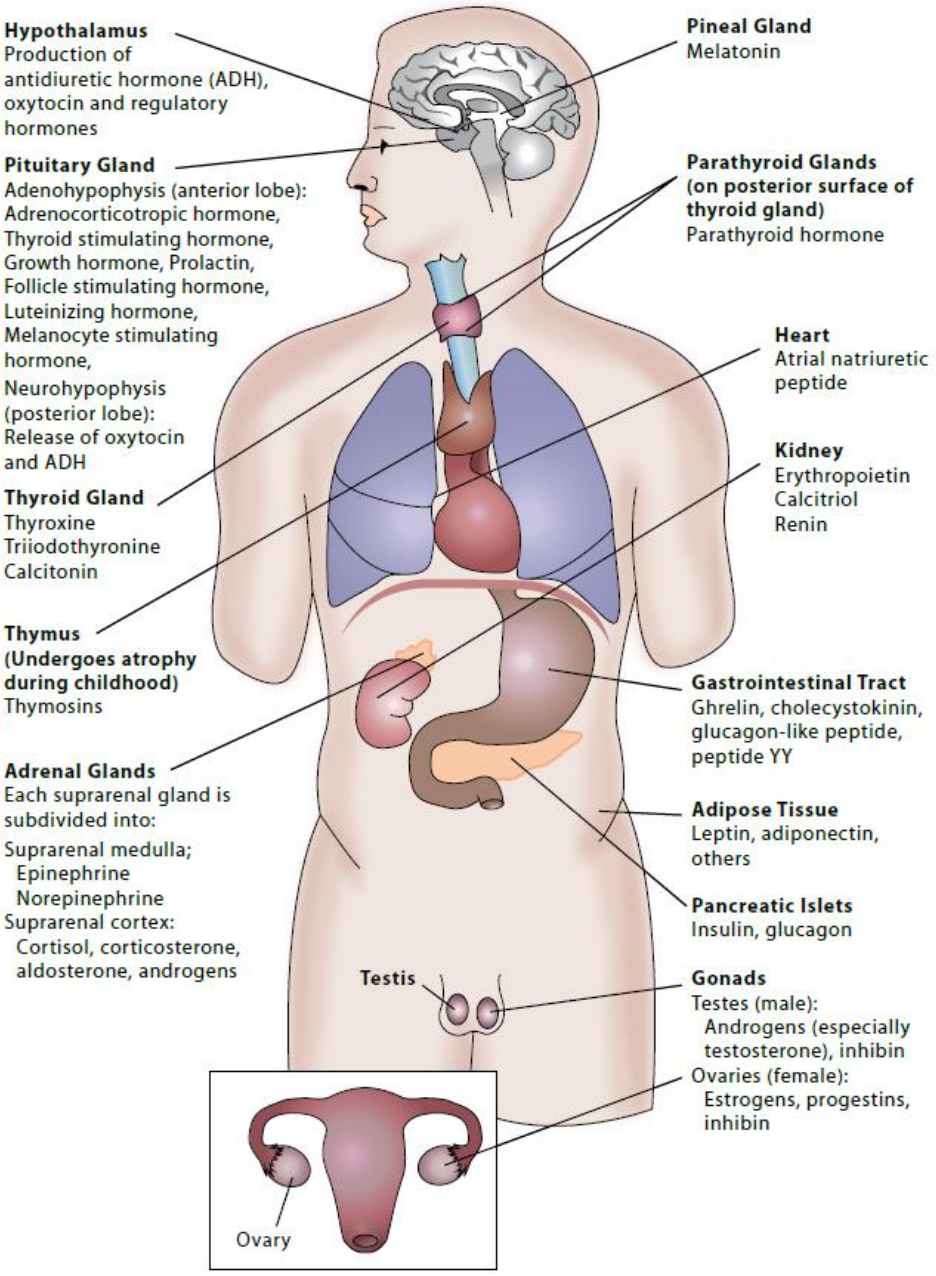


Figure 1.1 *An overview of hormone producing glands, organs and tissues* (from WHO/UNEP, 2013).

Because of its critical physiological functions, disruption to the endocrine system by altering the availability of a hormone or its action at a target site can have severe effects on an organism, some of which can be irreversible. Indeed, a number of endocrine disorders have been observed in humans, which can cause abnormal development. For example, congenital hypothyroidism can cause neurological damage, including intellectual deficits and cretinism, if it is not identified and treated early

(Zoeller *et al.*, 2002). Similarly, deficiency in the enzyme 5α-reductase, which converts testosterone to the more potent 5α-dihydrotestosterone (DHT), can result in ambiguous external genitalia, micropenis, prostate hypoplasia, reduced spermatogenesis and scarce facial and body hair (Cheon, 2011). In addition, disruption to homeostatic regulation can have serious implications for survival, such as insulin resistance occurring in type 2 diabetes and cases of obesity, which results in a loss of control of glucose regulation and hypoglycaemia. As a result, diabetic individuals are more likely to be afflicted with heart disease, kidney failure, limb amputation and blindness (Smyth and Heron, 2006).

Hormones bind to specific receptors with a high affinity to become signal transduction systems mediating hormonal responses (Welshons *et al.*, 2003). Hormone receptors are found on cell membranes or within the cell cytoplasm or nucleus. Hydrophilic hormones, such as hypothalamic and pituitary hormones, are not lipid soluble and are therefore unable to pass through the cell membrane. Instead, they bind to receptors on the cell membrane and stimulate or inhibit a second messenger system. This induces a cascade of protein phosphorylations, producing transcription factors to allow the hormone to indirectly regulate the transcription of its response genes. In contrast, lipophilic hormones, such as the sex steroids, can pass through the cell membrane to bind to intracellular receptors. The hormone-receptor complex then becomes a transcription factor itself by binding to a hormone response element on the DNA within the nucleus of a cell to activate transcription (Welshons *et al.*, 2003). These genomic responses are slow and can take a number of hours due to the time taken to synthesise and accumulate the protein (Schmidt *et al.*, 2000), although faster responses can be achieved via the membrane receptors. Indeed, there is now a large body of evidence to suggest that lipophilic steroid hormones can also act on receptors in the membrane to induce these fast responses (Falkenstein *et al.*, 2000). For example, aldosterone can rapidly activate ion transport mechanisms to cause changes in cell volume (Lösel and Wehling, 2003).

Due to the high affinity and specificity between receptors and their hormone ligands, they are capable of producing a response at the low concentrations at which they are present in the blood (pg/mL) (Vandenberg *et al.*, 2012). In addition, the number of receptors with bound hormones, receptor occupancy, has a non-linear relationship with the concentration of available hormones (Figure 1.2), which can bind up to the point of receptor saturation when a maximal response is reached. As a result, a small change in hormone concentration at the low end will have a much greater impact on receptor occupancy than a change at the high end of the concentration range. In most cases,

fewer than 5% of all receptors are bound to their ligand, meaning that the system is prepared to have large effects from small changes in hormone concentrations (Vandenberg *et al.*, 2013; Nussey and Whitehead, 2001). Receptor occupancy also has a non-linear relationship with the response, in part due to the saturation of the response at lower concentrations than those required to reach saturation of the receptor. This is termed the “spare receptor” hypothesis, which suggests that there are surplus receptors to those required to produce a biological response (Welshons *et al.*, 2003). Hormones have at least a sigmoidal relationship with the response, as shown in Figure 1.2, but in some cases non-monotonic dose-responses can also occur, in that the slope of the dose-response curve changes from positive to negative or vice versa (Kohn and Melnick, 2002). This can result from a reduction in the abundance of receptors as a result of hormone mediated downregulation, degradation, or desensitisation of the receptor to reduce its binding affinity (Vandenberg *et al.*, 2012). When these receptor interactions occur at higher concentrations than those that induce the maximal response, it results in a drop in the response curve.

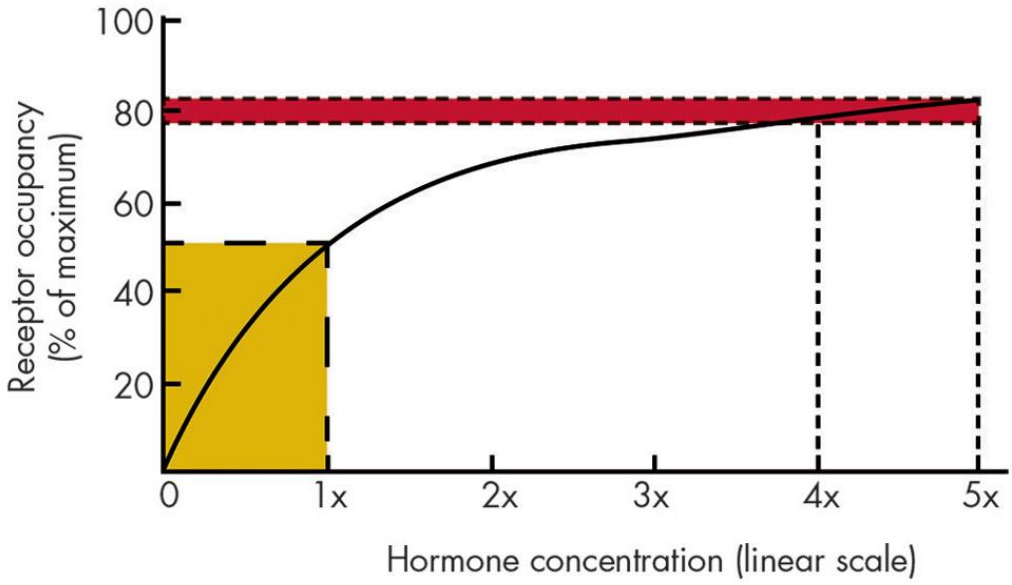


Figure 1.2 *A theoretical example of the non-linear, sigmoidal dose-response of hormone binding. At the low end, an increase in hormone concentration by 1x causes a 50% increase in receptor occupancy (yellow), in comparison to 4% at the high end (red)* (from Vandenberg *et al.*, 2012).

The presence of receptors within the endocrine system is subject to both spatial and temporal variation, with the magnitude of a biological response depending on the

number of available receptors, as well as the hormone concentration (Zoeller *et al.*, 2012). In some cases these receptors are expressed only in specific tissues, such as receptors for thyroid stimulating hormone, which are only found in the thyroid. Others, like the thyroid hormone receptor are found throughout the body (Vandenberg *et al.*, 2012; Cheng *et al.*, 2010). This spatial spread of receptors determines the scope for hormone action within the endocrine system, which can be broad, such as thyroid hormone or localised, such as thyroid releasing hormone. Temporal variation in the expression of receptors specifies hormone action at different stages of development as well as throughout the duration of physiological processes. Indeed, the expression of the gene coding for oestrogen receptor β was found to fluctuate during oestrus in the rat ovary (Hiroi *et al.*, 1999). In addition, some receptors have a number of different subtypes, which can have distinct roles and mediate different responses to a single hormone. For example, the thyroid hormone receptor has two isoforms, with receptor α regulating heart rate and receptor β modulating serum cholesterol, amongst other subtype specific effects (Lin *et al.*, 2013). In this case, analysis of thyroid hormone gene regulation failed to find any receptor specific differences, suggesting that their differential effects may be mediated by differences in the magnitude of the gene expression that each subtype could induce.

1.4 Endocrine disrupting chemicals and the endocrine system

To recap, an EDC is defined as "*an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations"* (International Programme on Chemical Safety, 2002). Early research on EDCs suggested that they exerted their effects through interaction with nuclear receptors, such as the androgen, oestrogen and thyroid receptors. However, in the last ten years it has been accepted that the scope for the action of EDCs is in fact much broader. It is now considered that they can exert their effects through direct action with hormone receptors and/or by controlling hormone delivery to the receptors (WHO/UNEP, 2013). This has opened all aspects of the endocrine system in both males and females to disruption by these exogenous chemicals, which can have serious physiological implications for both the development and function of an organism.

Direct genomic interactions of EDCs with hormone receptors can occur in an agonistic fashion to stimulate a transcriptional response in a hormone sensitive gene. In many cases these chemicals are less potent than natural hormones, in that greater

concentrations are required to induce a maximal response equal to that of the endogenous hormone. Indeed, xenoestrogenic EDCs, such as NP, OP and BPA, are 10,000 to 100,000 times less potent than endogenous E2, respectively. However, other chemicals can be considerably more potent, such as the pharmaceutical oestrogens diethylstilbestrol (DES) and EE2, that latter being ten times more potent than E2 in fish models (Caldwell *et al.*, 2012; Gutendorf and Westendorf, 2001). Non-genomic actions of EDCs have also been demonstrated but are less well studied, such as BPA binding to membrane receptors in pancreatic β cells to increase calcium ion oscillations and insulin secretion (Watson *et al.*, 2011; Nadal *et al.*, 2000). Other chemicals can interact antagonistically with the receptor, binding without activating a response and blocking the binding of endogenous hormone agonists to inhibit their action. In addition, some EDCs are only partial agonists to a receptor, in that they have a lower intrinsic activity and therefore produce a submaximal response compared with the endogenous hormone (Soto *et al.*, 2006). At appropriate concentrations, partial agonists can act in an agonistic fashion when there are sufficient spare receptors to bind to, but at higher concentrations they can also act as antagonists by blocking the binding of the more potent, natural ligands. This is highlighted by the pharmaceutical tamoxifen, which is used as an oestrogen receptor antagonist to treat breast cancer, although it is also a partial agonist. At therapeutic concentrations it can inhibit proliferation of oestrogen stimulated breast cancer cells, but it can also promote proliferation at lower sub-therapeutic concentrations, as a result of its weak agonistic activity. It therefore produces a “flare” in cancer cell proliferation early in treatment, before the therapeutic dose is achieved in the patient (Vandenberg *et al.*, 2012; Howell *et al.*, 2001). This produces a non-monotonic dose response curve, which will be further discussed. To further complicate the issue, single EDCs can interact with the endocrine system through multiple mechanisms involving multiple receptors. For example, some pesticides and plasticisers act as both androgen receptor (AR) antagonists and oestrogen receptor agonists (Kojima *et al.*, 2004; Sohoni and Sumpter, 1998). Some parent compounds can also break down to produce active metabolites, such as the anti-androgenic DDT metabolite *p,p'*-DDE (Kelce *et al.*, 1995). In addition, disruption of hormone synthesis, transport and elimination can reduce hormone concentrations at the target receptor. By limiting any or all of these factors, the amount of hormone available to bind to the receptor will decrease, reducing the magnitude of the response. In the case of the phthalate ester DEHP, exposure of male rats during foetal life causes reproductive tract malformations due to the suppression of testosterone concentrations. This is thought to be caused by a reduction in cholesterol uptake and steroid synthesis (Borch *et al.*, 2006).

Since EDCs can act like hormones, it has been proposed that testing and regulation of these substances adheres to principles of endocrinology (Vandenberg *et al.*, 2013; Zoeller *et al.*, 2012; Diamanti-Kandarakis *et al.*, 2009). This stance has caused much debate amongst researchers and regulators since this viewpoint contrasts with the long held dogma in toxicological testing of “the dose makes the poison” (Holmes, 2013). This principle assumes that the effects of low doses can be predicted from the effects of high doses, based on a linear, monotonic relationship (Myers *et al.*, 2009; Hotchkiss *et al.*, 2008). Low doses have been defined as either an environmentally relevant dose or a dose below the concentration at which a biological effect occurs in traditional toxicological tests (Melnick *et al.*, 2002). However, research suggests that, like hormones, EDCs can act at low doses and produce non-linear or even non-monotonic dose response curves, making the effects of low doses difficult to predict. As discussed, the non-linear relationships between hormone and receptor and receptor and effect (Figure 1.2) mean that the endocrine system is very sensitive to changes at the low end of the dose response curve, where it can produce a large response (Vandenberg *et al.*, 2013; Nussey and Whitehead, 2001). EDCs obey the same rules when interacting with receptors and so some can also induce effects at low concentrations (Vandenberg *et al.*, 2012; Welshons *et al.*, 2003). Some EDCs also have a high affinity to hormone receptors, such as EE2, which preferentially binds with a high affinity to oestrogen receptor α (Gutendorf and Westendorf, 2001). Other proposed mechanisms for low dose effects include additive action with endogenous hormones, greater bioavailability of EDCs, which show low binding affinity to high affinity protein transporters, and changes in receptor abundance (Vandenberg *et al.*, 2012). Some examples of EDCs which can exhibit low dose effects were highlighted in a review by an expert panel from the US National Toxicology Program and include: DES, methoxychlor, NP and genistein (Melnick *et al.*, 2002). However, a more recent review has found evidence for low dose effects in a more diverse set of chemicals, including industrial chemicals, plasticizers, pesticides, phytoestrogens, preservatives, surfactants and flame retardants (Birnbaum, 2012; Vandenberg *et al.*, 2012).

As well acting in a non-linear fashion, non-monotonic dose response curves also seem to be common amongst EDCs. These have been reviewed by Vandenberg *et al.* who collected several hundred examples of non-monotonic dose response curves reported in cell lines, whole organisms and human populations (Birnbaum, 2012; Vandenberg *et al.*, 2012). Multiple mechanisms have been proposed to explain this phenomenon, which can produce “U shaped” or “inverted U shaped” curves as well as other curves which can be more complex. These include cytotoxicity at high concentrations, which

negatively impact the positive endocrine response achieved at lower concentrations, particularly in studies employing cell lines (Welshons *et al.*, 2003). Changes in the association between an EDC and the receptor from agonism at low doses to antagonism at high doses, such as in the case of tamoxifen, can produce similar effects (Howell *et al.*, 2001). Alternatively, an EDC may act on multiple receptors at high concentrations to produce different responses through multiple gene pathways (Kojima *et al.*, 2004). Changes in receptor abundance with up-regulation at low concentrations and downregulation or desensitization at high concentrations can also occur (Vandenberg *et al.*, 2012). Whilst it is clear that replicable non monotonic dose response curves occur through exposures to EDCs, more work is needed to elucidate the exact endocrine mechanisms through which this occurs for specific chemicals. In addition, testing should ensure that a sufficient range of doses are employed to ensure that significant adverse effects are detected (Myers *et al.*, 2009). This is highlighted by an *in utero* exposure of rats to DEHP, where brain aromatase was inhibited at low doses, below the perceived no observed effect concentration (NOEC), and stimulated at high doses in males on postnatal day 1 (Andrade *et al.*, 2006). This is displayed in Figure 1.3.

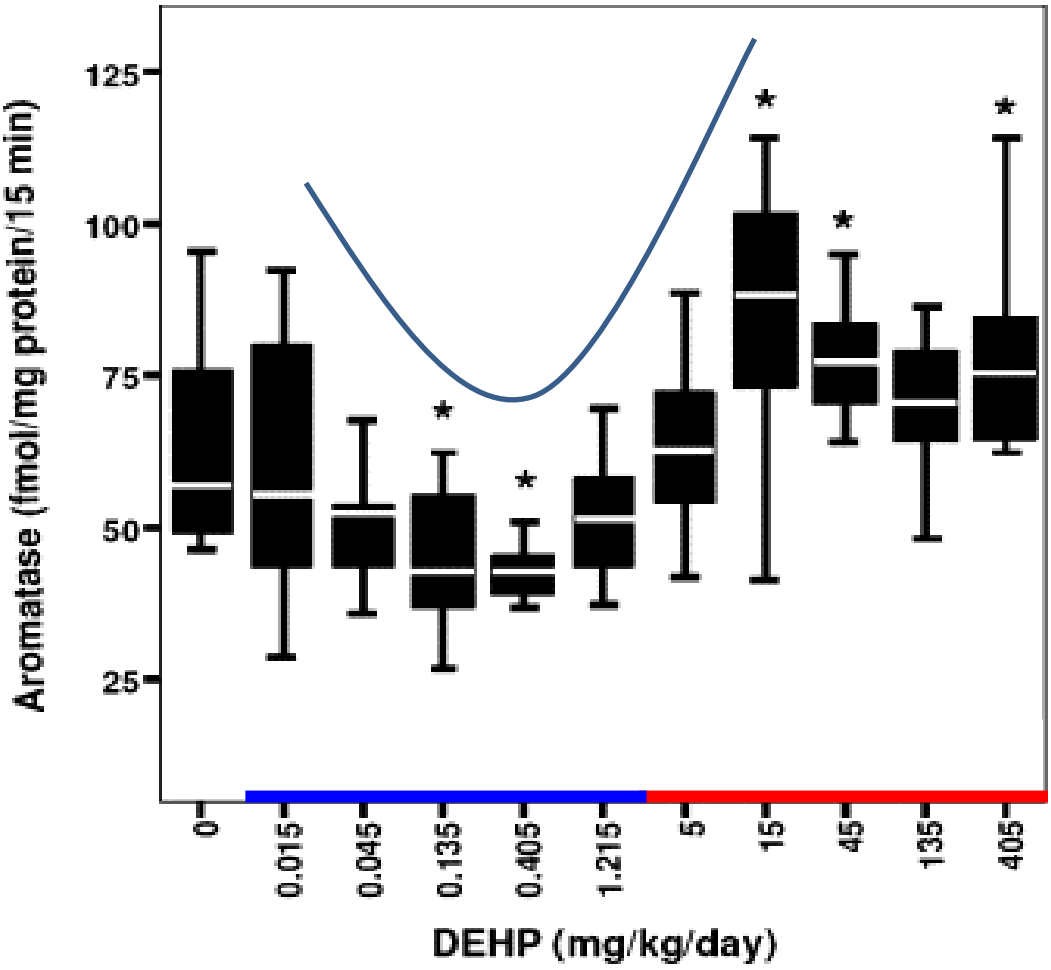


Figure 1.3 *The effects of DEHP on hypothalamic/preoptic area aromatase activity in male rats at post natal day one. The box plots indicate medians (horizontal lines) with 25 and 75% quartiles and error bars extending to the maximum and minimum values. * indicates a significant difference from the control group. The low dose region [–] started at a similar concentration to the estimated median intake of the general German population* (Koch *et al.*, 2003)*. The high dose region [–] ended with the highest dose at 405 mg/kg/d, which is known to cause reproductive defects without maternal toxicity. The inverse U shape of the response curve is also displayed* (adapted from Andrade *et al.*, 2006)*.*

Additional observations of the differential effects of chemicals at different life stages further contrasts with the "dose makes the poison" principle. The effects of EDCs depend on the stage of development of an organism and the timing of the exposure within its life cycle, where some developmental stages may be more sensitive to perturbation than others (Zoeller *et al.*, 2012). Like natural hormones, this can potentially induce irreversible changes if exposures occur during sensitive "critical

windows," such as when organs are forming or differentiating. For example, an androgen programming window has been identified in rats during foetal development when androgens induce masculinisation of the reproductive tract. It was only by exposing males during this specific time point that disrupting endogenous androgens with anti-androgenic chemicals could cause reproductive tract malformations and a reduction in penile length (Welsh *et al.*, 2008). Indeed, disruption during the sensitive period of early development may also have adverse effects that only present themselves in later life. This is a well-known concept in human health termed the "foetal basis of adult disease," which linked conditions such as coronary heart disease and type 2 diabetes to undernutrition during foetal life (Barker and Clark, 1997). It is supported by life history theory, which suggests that undernutrition leads to a reduction in the available energy allocation and can permanently alter the body's structure, function, and metabolism (Barker, 2004). One of the best examples in human health is the case of the pharmaceutical DES, which was prescribed to pregnant women to prevent miscarriage between 1940 and 1971. As well as causing reproductive abnormalities, the *in utero* exposure of DES daughters was linked to clear cell adenocarcinoma and increased likelihood of miscarriage, ectopic pregnancies and premature births in later life (Hotchkiss *et al.*, 2008). In addition, there is increasing concern for a role of EDCs in the obesity epidemic, which cannot be solely explained by overeating and inactivity (Baillie-Hamilton, 2002). A number of chemicals, including DES, BPA and organotins, have been found to disrupt the normal developmental and homeostatic controls over adipogenesis and energy balance. This has led to weight gains in experimental animals following *in utero* exposures (Newbold *et al.*, 2008; Grün and Blumberg, 2006). In addition, a recent epidemiology study has linked prenatal exposure of PCBs and DDT to obesity in the daughters of women from the Faroe Islands in Denmark (Tang-Péronard *et al.*, 2014).

Advances in research also suggest that the effects of exposure to EDCs can be more long term than just the lifetime of an exposed individual, as evidence of transgenerational effects is growing. Indeed, maternal exposure not only affects the mother (F0), but also the embryo (F1) and the F2 generation present as germ cells within the embryo (Patisaul and Adewale, 2009). One mechanism by which transgenerational effects can occur is through epigenetic changes in the germ line. These are inheritable changes in gene expression patterns by molecular processes such as DNA methylation or histone modification, which can occur without changing the gene sequence itself. Indeed, methylation of a gene promoter region can reduce transcription, whilst acetylation of the histone tail can have a promoting effect (Gore,

2008). This has been observed in exposures of rats to the anti-androgenic vinclozolin or oestrogenic methoxychlor pesticides during foetal development. Here, a spermatogenic cell defect leading to subfertility in F1 males was transferred to subsequent generations, from F1 to F4, and correlated with DNA methylation in the germ line (Anway and Skinner, 2006; Anway *et al.*, 2005). DES has also demonstrated inheritable traits in rodent studies, where the offspring of rodents exposed *in utero* were found to be more susceptible to tumour formation. Again, this was thought to occur through epigenetic mechanisms and has caused concern for DES granddaughters (Newbold *et al.*, 2006).

1.5 Endocrine disruption in humans and wildlife

In the last few decades, increased research on endocrinology and toxicology has expanded the scope of endocrine disruption to encompass the entire endocrine system. There is also increasing evidence for long term impacts of EDCs, low dose effects, and the combined action of mixtures. Consequently, the implication of chemical pollutants in the aetiology of endocrine disorders in humans and wildlife is an increasing cause for concern. The research contributing to this thesis surrounds endocrine disruption specifically in terms of reproductive effects in fish and therefore the bulk of this section mainly focuses on sexual disruption in fish. However, it should be recognised that endocrine disruptive effects have been observed across a wide variety of vertebrate and invertebrate taxa and there are an increasing number of cases in which effects in wildlife can be attributed to EDCs (WHO/UNEP, 2013).

1.5.1 Endocrine disruption in humans

Research suggests that like other vertebrates, humans are also susceptible to endocrine disruption by exogenous chemicals. This is highlighted by the case of DES, which failed to fulfil its purpose of preventing miscarriage and was found to cause reproductive disorders and cancers in children born to DES mothers. Indeed, clear cell adenocarcinoma, abnormalities of the cervix, uterus and fallopian tubes, as well as increased likelihood of miscarriage, ectopic pregnancies and premature birth were observed in daughters (Hotchkiss *et al.*, 2008). Effects were also reported in sons, including hypospadias (the abnormal placement of the male urethral orifice) and cryptorchidism (undescended testes), as well as reductions in sperm count, the number of motile sperm and sperm with normal morphology, although fertility appeared

unaffected (Toppari *et al.*, 1996). The link to these disorders is supported by studies in rodents, where similar effects were observed, such as cancers, reduced fertility and reproductive tract malformations (Newbold, 2001). These studies also demonstrated transgenerational inheritance of cancers through epigenetic changes in the maternal germ line, which has caused concern for the health of DES granddaughters (Newbold *et al.*, 2006). In addition to DES, some environmental chemicals have also been associated with endocrine disorders following occupational exposures or poisoning events. For example, cryptorchidism in male newborns has been associated with occupational pesticide exposure of mothers involved in agriculture and gardening in Norway and Denmark (Weidner *et al.*, 1998; Kristensen *et al.*, 1997). Hypospadias and other congenital anomalies have been associated with proximity to hazardous waste sites in Europe (Dolk *et al.*, 1998), whilst in Minamata, Japan, methylmercury poisoning of residents consuming contaminated fish resulted in severe neurological problems (Ekino *et al.*, 2007).

Like wildlife populations, humans globally are constantly exposed to pollutants in food, soil, water, air and dust, which can enter the body by inhalation, ingestion and dermal contact (WHO/UNEP, 2013). In 1951, the detection of DDT in human fat and milk was first reported in non-occupationally exposed mothers (Laug *et al.*, 1951). Since then it has become increasingly appreciated that the general human population has a body burden of a wide range of contaminants, which has led to an increase in biomonitoring. Of particular concern is the presence of these contaminants in pregnant women, since chemicals are capable of crossing the placenta into the foetus and have been detected in amniotic fluid, umbilical cord blood and meconium (Woodruff *et al.*, 2011; Barr *et al.*, 2007). Indeed, the ubiquitous exposure to some environmental chemicals was demonstrated in the US Health and Nutritional Examination Survey conducted from 2003-4. Here, certain PCBs, organochlorine pesticides, perfluorinated compounds, phenols, PBDEs, phthalates, polyaromatic hydrocarbons (PAHs), and perchlorate were detected in 99–100% of the 268 pregnant women studied (Woodruff *et al.*, 2011). Some of the chemical classes detected in a subsample of this study are shown below in Figure 1.4.

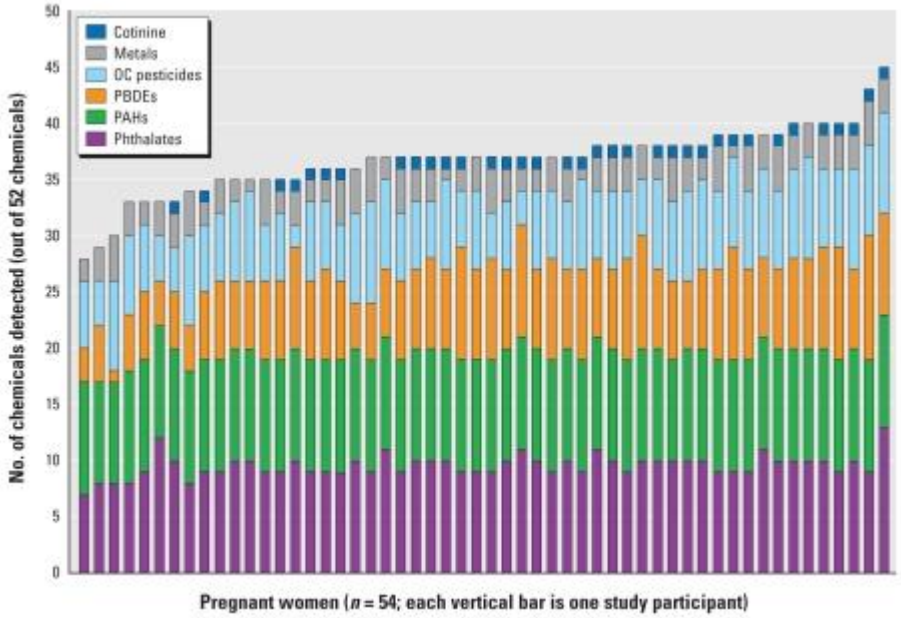


Figure 1.4 *The number of chemicals detected by chemical class in a subsample of 54 pregnant US women from the Health and Nutritional Examination Survey conducted from 2003-04* (from Woodruff *et al.*, 2011)*. Each vertical bar represents one study participant.*

Although much of the evidence for endocrine disruption in humans comes from accidental or occupational exposures to adverse chemicals, such as DES, there is evidence for increasing trends in hormone related disorders in the general human population. Indeed, EDCs may be contributing to the increasing trends in reproductive disorders, hormone related cancers, neurobehavioral disorders and even obesity, which have been observed in a number of countries in the last century (WHO/UNEP, 2013). In males, increasing trends in the incidence of testicular germ cell cancers, cryptorchidism and hypospadias, as well as reductions in semen quality have been observed in a number of countries and are well characterised in Europe (Jørgensen *et al.*, 2006; Richiardi *et al.*, 2004; Sharpe and Irvine, 2004; Toppari *et al.*, 2001). Since these disorders have been associated as risk factors of one another, they have been hypothetically linked as symptoms of a single, underlying condition termed testicular dysgenesis syndrome (Skakkebæk *et al.*, 2001). In humans, the symptoms may vary dependant on the severity of the condition and although genetic factors can be involved, a majority of newborns with malformations of the genitalia were found to have no known genetic defects. This, combined with the speed of increased incidence of these symptoms, suggests that lifestyle and environmental factors, such as EDCs, are

likely to be involved (Skakkebæk *et al.*, 2001). In fact, testicular dysgenesis syndrome is thought to have a foetal origin, resulting from disruption to prenatal testicular development during the androgen programing window. This is the point during foetal development in which androgens induce masculinisation of the reproductive tract and when disruption of androgen control can lead to abnormal development (Welsh *et al.*, 2008). Indeed, in mammalian studies, disruption of androgen production and/or androgen action at the receptor by EDCs has been shown to induce these disorders in the male offspring of exposed pregnant females (Wilson *et al.*, 2008; Earl Gray Jr. *et al.*, 2006). For example, di (*n*-butyl) phthalate caused a dose dependent induction of cryptorchidism, hypospadias and impaired spermatogenesis (Sharpe and Skakkebaek, 2008). In humans, prenatal exposure to phthalates, BPA and *p,p'*-DDE were also associated with lower ano-genital distance (Miao *et al.*, 2011; Torres-Sanchez *et al.*, 2008; Swan *et al.*, 2005). This measurement is normally higher in males than females and so a decrease indicates demasculinisation. As a result, whilst it was originally considered that these effects could be a result of exposure to environmental oestrogens, it now seems more likely they are caused by disruption of the oestrogen-androgen balance. Critically, the increase in focus on the role of androgens also implicates environmental anti-androgenic chemicals, which are capable of disrupting endogenous androgens (Sharpe, 2003). From this perspective, a cumulative risk assessment of 15 AR antagonists, including pesticides, phthalates and parabens was conducted to determine their possible impact on human reproductive health. Based on the median intake of these chemicals in the general human population and the concentrations required to cause adverse effects in rodent models, this study found that adverse effects were unlikely to occur in the general human population. In comparison, for individuals at the high end of the exposure range the cumulative risk from the combination of these chemicals was determined to exceed an acceptable level (Kortenkamp and Faust, 2010). However, when a mixture of 22 AR antagonists were combined at levels observed in human plasma, they failed to produce an anti-androgenic effect *in vitro*. This indicates an explanation gap between the cause and effects of testicular dysgenesis syndrome, which suggests that other as yet unknown chemicals would need to be contributing to the observed effects (Kortenkamp *et al.*, 2014).

Adverse female reproductive health trends include increases in the incidence of endometriosis, the preterm birth rate, uterine fibroids and difficulty in achieving or maintaining pregnancy, as well as the earlier onset of puberty (WHO/UNEP, 2013). As with male reproductive health issues, it is thought that EDCs could play a role in

inducing these effects through disruption of the hormonal control of development and function of the female reproductive system. Indeed, the human body burden of some pesticides has been associated with shorter menstrual cycle, earlier age of menarche and later menopause (Farr *et al.*, 2006; Ouyang *et al.*, 2005; Farr *et al.*, 2004). Exposure to *p,p'*-DDE in early life is also considered to be a risk factor for breast cancer in later life (Cohn *et al.*, 2007). In addition, thyroid disruption and effects on neurodevelopment in males and females have also been observed in animal models following exposure to chemicals such as brominated flame retardants, PBDEs, BPA and PCBs (Boas *et al.*, 2012; Zoeller, 2010; Legler, 2008). There is concern that these could be linked to increasing incidence of attention deficit/hyperactivity disorder (ADHD). Indeed, in the US there is evidence that children with higher serum concentrations of polyfluoroalkyl chemicals, such as PFOS and PFOA, have greater odds of having ADHD (Hoffman *et al.*, 2010). There is also evidence of a link between organochlorines and behavioural deficits in children following prenatal exposure (González-Alzaga *et al.*, 2013).

1.5.2 Endocrine disruption in wild vertebrates

Endocrine disorders have also been observed in a variety of wild vertebrate species, some of which show similarities to those observed in humans. Genital abnormalities and altered secondary sexual characteristics have been observed in wildlife in contaminated areas, causing concern for impacts on reproductive fitness. For example, abnormal ovarian morphology, increased E2 concentrations and multioocyte follicles have been identified in female American alligators (*Alligator mississippiensis*) in Lake Apopka (Milnes and Guillette Jr., 2008). Reproductive success in this population is low and a loss of sexually dimorphic gene expression has been observed (Milnes *et al.*, 2008). In males from this site, a reduction in phallus size compared with control lakes has also been observed. Similarly, reduced baculum (penile bone) size has been observed in otters in the UK and Canada, where baculum size was negatively correlated to hepatic organochlorine concentrations in both countries (Grove and Henny, 2008; Simpson, 2007). The body burdens of these chemicals have also been correlated with uterine obstructions and reduced pregnancies in Baltic grey seals *(Halichoerus grypus*) (O'Hara and Becker, 2003) and organohalogens have been negatively correlated with the size of both the baculum and testes in male polar bears and the ovaries and reproductive tract in females (Sonne *et al.*, 2012). Cryptorchidism, occurs with abnormally high incidence in a population of sitka black-tailed deer (*Odocoileus hemionus sitkensis*) on Kodiak Island in Alaska, and was associated with

antler abnormalities (Veeramachaneni *et al.*, 2006). In other cases where cryptorchidism is abnormally high, including that of the Florida panther, low genetic diversity and inbreeding may be a contributing factor. However, in the case of the Kodiak Island deer, the data favours an impact of environmental contaminants on embryonic development since there is no evidence of excessive inbreeding or genetic drift (Latch *et al.*, 2008). Nonetheless a genetic vulnerability in this population to a contaminant remains plausible and with a majority of the effected animals being reproductively sterile, there is a risk of population level effects and a reduction in genetic diversity (Veeramachaneni *et al.*, 2006).

Intersexuality, the simultaneous presence of male and female tissue within the gonad of an individual, has also been observed in amphibian populations globally, where it is also considered to be a feminisation response in males. In Canada, the occurrence of intersex in two frog species has been associated with agricultural areas and was correlated with the presence of pesticides (McDaniel *et al.*, 2008). Indeed, historical data on intersex in cricket frogs (*Acris crepitans*) in the US suggests that the greatest incidence occurred in periods associated with high use of DDT and PCBs (1946-1959). Incidence declined as the use of these chemicals and their environmental presence reduced from 1960 onwards. However, in other localities, higher incidence of intersex in frogs has been observed in urban and industrial areas compared to agricultural areas, suggesting that there are likely to be multiple chemical causes (Skelly *et al.*, 2010; Reeder *et al.*, 2005). Intersex has also been observed in reptiles, such as red bellied turtles (*Pseudemys nelsoni*) in the pesticide contaminated Lake Apopka, Florida (Guillette Jr. *et al.*, 1995). There is also evidence of intersex in birds, although this could be occurring naturally since it seems to regress and was not present in 21 day old hatchlings from a PCB contaminated site (Hart *et al.*, 2003). In mammals, true hermaphroditism has been discovered in a beluga whale (*Delphinapterus leucas*), with two testis and two ovaries, in the St Lawrence estuary, Canada (De Guise *et al.*, 1994). In addition, one case of ova-testes intersex has been observed in a stranded common dolphin (*Delphinus delphis*), in which ovarian tissue was developing normally and the testicular tissue was degenerating (Murphy *et al.*, 2011). Pseudohermaphroditism has also been identified in other cetaceans (Tarpley *et al.*, 1995), as well as polar bears (*Ursus maritimus*) (Wiig *et al.*, 1998), American black bears (*Ursus americanus*) and brown bears (*Ursus arctos*) (Cattet, 1988). The incidence of these abnormalities in the tested populations seems to be considerably lower than that of intersex in wild fish, although this could be affected by sampling much fewer individuals. For example, the study of abnormalities in black bears and brown bears only assessed 38 and four

individuals respectively, of which abnormal sexual differentiation was observed in four black bears and one brown bear. In addition, in polar bears, female pseudohermaphroditism was only observed in four out of 269 individuals (Wiig *et al.*, 1998).

An indicator of feminisation is the presence of an abnormally high blood plasma concentration of vitellogenin in male oviparous vertebrates. High concentrations of vitellogenin are normally detected in females since it is an egg yolk precursor molecule, but its production can also be induced in males by EDCs. Indeed, vitellogenin induction in male peregrine falcons (*Falco peregrinus*) in Spain was hypothesised to be linked to organochlorine chemicals (e.g. PCBs and DDTs). However, the implications of this are as yet unknown and since the peregrine falcon is on the International Union for the Conservation of Nature's red list as "threatened," negative impacts of pollutants remain a cause for concern (Jiménez *et al.*, 2007). There is also some evidence of altered vitellogenin concentrations in reptiles at contaminated sites, including male snapping turtle (*Chelydra serpentina*) in Canada (Environment Canada, 2003) and female freshwater turtles (*Chrysemys picta*) in North America (Rie *et al.*, 2005). However, this is not as well researched as vitellogenin induction in wild fish, which will be further discussed.

One of the most notable cases of a set of EDCs adversely affecting wildlife is the case of organochlorine pesticides and their link to widespread population declines in higher trophic level birds (Lundholm, 1997). Following the onset of prevalent organochloride use in the late 1940's, adult mortality due to accumulated pesticide residues and nest failure through eggshell thinning were first observed in North America, in species including bald eagles (*Haliaeetus leucocephalus*), osprey (*Pandion haliaetus*) and peregrine falcon (Henny *et al.*, 2010; Brown *et al.*, 2007). Similarly, in the UK incidence of broken eggs in the nests of predatory birds such as sparrowhawk (*Accipiter nisus*) and golden eagle (*Aquila chrysaëtos*) increased considerably between 1951 and 1966 (Ratcliffe, 1967). This is thought to occur due to the inhibition of prostaglandin synthesis in the egg shell mucosa by *p'p*-DDE, which in turn reduces calcium transport to the egg shell (Lundholm, 1997). In some species over a 19% reduction in egg shell thickness was observed, which was associated with organochlorine residues and resulted in increasing egg breakage and eventually population declines (Hickey and Anderson, 1968). Peregrine falcons, for example were completely extirpated from the eastern part of the US by 1964 (Clark *et al.*, 2009). Following the ban on DDT in western countries in 1972, as well as human intervention with breeding programs and reduced persecution, many species have now recovered (Henny *et al.*, 2010; Brown *et*

al., 2007). There is now evidence for trends of increasing egg shell thickness, although organochlorine residues and also PCBs still continue to be detected and remain negatively correlated with shell thickness (Clark *et al.*, 2009).

As in humans, endocrine disorders in vertebrates which have been linked to EDCs are not limited to the reproductive system. Abnormalities indicative of thyroid disruption have been observed in marine mammals and linked to exposure to organochlorines, such as fibrosis of the thyroid gland in harbour porpoises (*Phocoena phocoena*) (Schnitzler *et al.*, 2008). Similarly, lesions of the thyroid gland were found in beluga whales from St Lawrence estuary and Hudson's Bay, Canada, which were unique to these populations (Mikaelian *et al.*, 2003). Enlarged thyroid glands were observed in herring gulls at sites highly contaminated with PCBs in the Great Lakes, Canada, in what is thought to be a response to reductions in available thyroid hormone (McNabb and Fox, 2003). Furthermore, disruption to thyroid hormone, vitamin D, calcium and phosphate, which are linked to bone homeostasis, have been correlated with hepatic PCBs and DDT in Baltic grey seals. This is thought to be linked to bone abnormalities including skull lesions and reduced bone density (Routti *et al.*, 2008). Impacts on the immune system have also been linked to pollutants, where PCB concentrations in the blubber of harbour porpoises were significantly higher in individuals that died through infectious disease in comparison to mortality through physical trauma (Jepson *et al.*, 2005). PCBs and other organochlorines are also hypothesised to have played a role in the phocine distemper virus outbreaks in European seal species. Indeed, at some sites higher concentrations of organochlorines were detected in the bodies of harbour seals (*Phoca vitulina*) that had died during the zoonotic compared to the survivors (Hall *et al.*, 1992). So far a causal relationship has not been firmly established (Härkönen *et al.*, 2006), although seal populations have been rising as POPs in the environment have reduced (WHO/UNEP, 2013). In addition, PAH exposure has been suggested as a significant cause of the abnormally high cancer rate identified in beluga whales in the St Lawrence estuary, Canada. Indeed, 27% of the stranded whales analysed contained cancerous tissue, which is the highest incidence in any cetacean population ever recorded (Martineau *et al.*, 2002). Similarly, the concentration of PCBs in the blubber of California sealions (*Zalophus californianus*) has been linked to the probability of the animals dying of cancer. Here, PCBs concentrations were 85% higher in the blubber of sealions affected with carcinoma (Ylitalo *et al.*, 2005).

1.5.3 Endocrine disruption in wild fish

Endocrine disruption in fish has become one of the best researched areas of the field, following the identification of widespread reproductive abnormalities in wild fish populations. This was particularly well characterised in the UK, where abnormally high concentrations of vitellogenin were observed in male fish caged downstream of WWTW effluent outfalls in the 1990's. This effect persisted for several kilometres downstream of the outfalls and was thought to be indicative of a feminisation response to oestrogenic EDCs originating from the WWTW effluent (Harries *et al.*, 1997; Purdom *et al.*, 1994). Further sampling of wild roach (*Rutilus rutilus*) in rivers across the UK also identified vitellogenin induction in males, which varied between sites and occurred at the highest concentrations downstream of wastewater treatment works (WWTWs). Critically, surveys also identified the widespread, abnormal occurrence of intersex in male fish, characterised by mixtures of both ovarian and testicular germ cells in the gonads and by the development of ovarian ducts (ovarian cavities) (Jobling *et al.*, 2006; Jobling *et al.*, 1998). At sites with high incidence of intersex the number of normal males was low, which in conjunction with the observed vitellogenin induction, suggested that this was also a feminisation response to effluent contamination. Indeed, the incidence of intersex was found to be correlated with the proportion of effluent in the river. In control sites with little effluent contamination, intersex still occurred but at a much lower rate of 4-18% compared with 16-100% at contaminated sites, which could suggest a low natural occurrence or another pollution source. However, roach are a dioecious species (Jafri and Ensor, 1979) and a study of their reproductive cycle at a site where oestrogenic activity was not detected found no cases of intersex in the 474 fish sampled. This suggested that intersex does not occur naturally in this species (Geraudie *et al.*, 2010).

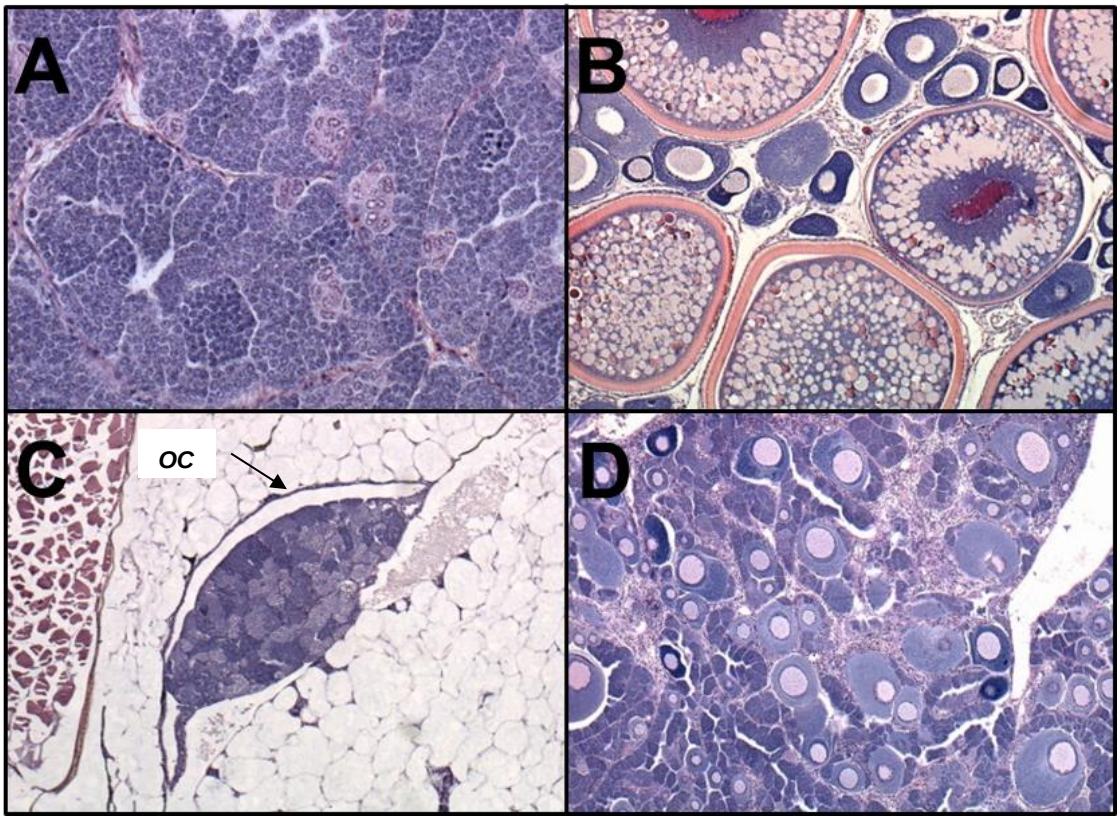


Figure 1.5 *Sexual disruption observed in the gonads of roach (Rutilus rutilus). Normal male testicular tissue (A); normal female ovarian tissue (B); an ovarian cavity (OC) in the testis of a juvenile roach exposed to oestrogenic WWTW effluent (C)* (from Baynes *et al.*, 2012)*; abundant testicular oocytes in a fish from an effluent contaminated river site in the UK (D).*

Intersex (as shown in Figure 1.5) and elevated vitellogenin has since been identified in other fish species in the UK, such as the gudgeon (*Gobio gobio*) (van Aerle *et al.*, 2001), as well as in freshwater species in European countries including France (Maltret-Geraudie *et al.*, 2008; Minier *et al.*, 2000), Germany (Hecker *et al.*, 2002), Denmark (Bjerregaard *et al.*, 2006), Czech Republic (Randak *et al.*, 2009), Spain (Solé *et al.*, 2003) and Italy (Viganò *et al.*, 2010). Intersex fish have also been reported further afield in North America (Hinck *et al.*, 2009; Vajda *et al.*, 2008; Woodling *et al.*, 2006), Japan (Hashimoto *et al.*, 2000), Brazil (De Sá *et al.*, 2008) and South Africa (Barnhoorn *et al.*, 2004). Species specific differences in the occurrence of these traits have also been demonstrated. For example, assessments of fish in the Great Lakes, Canada, found no gonadal abnormalities in goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*), gizzard shad (*Dorosoma cepedianum*), brown bullhead (*Ictalurus ameiurus*), pumpkinseed (*Lepomis gibbosus*), and bluegill (*Lepomis macrochirus*), but

did find widespread intersex (43-83% prevalence) and vitellogenin induction in white perch (*Morone americana*) (Kavanagh *et al.*, 2004). Furthermore, fish in the estuarine environment also seem to be affected, with elevated vitellogenin occurring in flounder (*Platichthys flesus*) and viviparous blenny (*Zoarces viviparus*) (Kirby *et al.*, 2004; Matthiessen *et al.*, 1998a). In several UK estuaries, a few sites also exhibited intersex in these species (Kirby *et al.*, 2004; Matthiessen *et al.*, 1998a). More recently, vitellogenin induction has also been observed in marine species including male cod (*Gadus morhua*) and dab (*Limanda limanda*) in the North Sea (Scott *et al.*, 2007; Scott *et al.*, 2006), swordfish (*Xiphias gladius*) and northern bluefin tuna (*Thunnus thynnus*) in the Mediterranean (Fossi *et al.*, 2006; Fossi *et al.*, 2004) and Chilean flounder (*Paralichthys adspersus*) in the South Pacific (Leonardi *et al.*, 2012), suggesting that the marine environment is also impacted by EDCs.

A concerning implication of sexual disruption is the possible impact at a population level since the intersex condition is associated with other reproductive problems in males, particularly in the most severe cases. Indeed, reductions in spermiation, milt volume, sperm density and gamete quality are associated with intersex in wild roach. Interestingly, the maturation of ovaries was less obviously effected in female fish sampled in the UK river surveys, although ovarian atresia was identified in fish from effluent contaminated sites (Jobling *et al.*, 2002b; Jobling *et al.*, 2002a). In addition, breeding studies using wild caught roach also demonstrated a negative correlation between intersex severity, fertilisation success and reproductive performance. However, reproductive performance only correlated significantly when severely intersex individuals were included, where a 76% reduction in reproductive performance was observed (Harris *et al.*, 2011). Assessing whether population level impacts are occurring in wild populations through reductions in reproductive fitness, individual survival or reductions in genetic diversity remains an important research question.

Other changes to genital structure and secondary sexual characteristics have also been reported in wild fish, causing concern for potential impacts on the reproductive fitness of these individuals. The development and morphology of these characteristics are hormonally controlled and are sexually dimorphic in nature, making them good biomarkers for the impacts of EDCs. For example, abnormalities indicative of feminisation in the morphology of the urogenital papilla, a copulatory organ, occurred in up to 75% of sampled male sand gobies (*Pomatoschistus minutus* and *P. lozanoi*) in UK estuaries associated with oestrogenic activity and vitellogenin induction in flounder (Kirby *et al.*, 2003). Similarly, in male mosquitofish (*Gambusia a. holbrooki*), reductions in the length of the gonopodium, a modified anal fin used in copulation, have been

associated with effluent contamination in Australia (Batty and Lim, 1999). This was also observed in mosquitofish in the pesticide contaminated Lake Apopka, Florida, alongside feminised American alligators, which also showed reductions in penile length (Toft *et al.*, 2003; Guillette *et al.*, 2000). Reduced secondary sexual characteristics of structures without direct reproductive function have also been observed in fathead minnows (*Pimephales promelas*), where reductions in the size of the dorsal fatpad and tubercles occurred in WWTW effluent contaminated rivers (Tetreault *et al.*, 2012). Although they play no role in copulation, the feminisation of these traits could still lead to reductions in reproductive fitness by reducing their ability to compete adequately for nesting sites and females. Conversely, the development of male secondary sexual characteristics in female fish can be attributed to masculinisation by EDCs. This has been observed in female mosquitofish, which have developed gonopodia downstream of androgenic pulp and paper mill effluents in a number of countries (Hewitt *et al.*, 2008). Indeed, in the Fenholloway River, Florida, assessment of gonopodium length in female mosquitofish found 80% of individuals to be partially masculinised and 10% to be completely masculinised (Parks *et al.*, 2001). Skewed sex ratios in favour of males have also been observed in viviparous eelpout (*Zoarces viviparus*) near pulp and paper mills in Sweden, in comparison to a set of reference sites where a stable sex ratio of around 50/50 had been observed over four years. In this case the sex ratio near the mill recovered following a 17 day shutdown, which coincided with the period of sexual differentiation in the development of the eelpout (Larsson and Förlin, 2002).

Non-reproductive effects of EDC contamination are less well studied in wild fish. Nonetheless, there is evidence of significantly reduced prey capture by mummichogs (*Fundulus heteroclitus*) in a mercury polluted tidal creek in New Jersey, USA (Smith and Weis, 1997). These fish made significantly fewer attempts to capture prey in comparison to those from a reference site, suffered significantly greater predation and had significantly higher concentrations of methylmercury in their brain tissue (Zhou *et al.*, 2000). These effects were thought to be mediated through disruption to the thyroid system, which has also been documented in walleye (*Sander vitreus*) in the Ottawa River (Picard-Aitken *et al.*, 2007). This was also observed in mummichog in San Francisco Bay where thyroid parameters were significantly correlated to hepatic PCBs (Brar *et al.*, 2010). In addition, fish chronically exposed to pollutants in the aquatic environment were also found to be incapable of eliciting a stress response through the elevation of cortisol in response to capture (Hontela *et al.*, 1995; Hontela *et al.*, 1992). In these cases, evidence suggested that this endocrine axis had reached an exhaustion phase due to prolonged hyperactivity. As a result, there were concerns for

effects on the survival of exposed individuals, since this response has been linked to immunosuppression and changes in behaviour.

Under laboratory conditions, exposures of fish to WWTW effluents for prolonged periods have been shown to cause other adverse health effects. In roach, these included alterations in kidney development, as well as genotoxic and immunotoxic effects, which occurred during exposure to lower effluent concentrations than those required to induce reproductive abnormalities (Liney *et al.*, 2006). Genotoxic effects have also been observed in carp exposed to water samples from the heavily polluted Noyyal River, India, where the number of DNA strand breaks increased with the duration of exposure (Rajaguru *et al.*, 2003). The immune system has also been demonstrated as a target for EDCs in a number of laboratory studies, and there is increasing evidence of an immune-endocrine system association. Indeed, during spawning in rainbow trout (*Oncorhynchus mykiss*) the decrease in immune parameters, such as the number of anti-body producing cells and plasma immunoglobulin concentrations, was correlated with plasma sex steroid concentrations (Hou *et al.*, 1999). Experimentally, leukocyte proliferation, macrophage activity and proteins relating to innate and acquired immunity can all be affected by EDCs. Interestingly, there is also evidence of positive effects on immune parameters, such as the stimulation of phagocytosis by some oestrogenic EDCs (Milla *et al.*, 2011). Some immune parameters are very sensitive to exposure to PAHs, which can cause an increase in the susceptibility of a fish to bacterial pathogens (Reynaud and Deschaux, 2006). Finally, there is also experimental evidence for neuroendocrine disruption in fish by EDCs, with pollutants affecting gonadotrophin releasing hormone (GnRH), as well as neurotransmitters, such as serotonin and dopamine. As a result, it is plausible that this could adversely affect development and the sexual differentiation of the brain (reviewed in Le Page *et al.*, 2011).

1.6 Sexual disruption in male fish: disruption to the HPG axis and the role of oestrogenic contaminants

1.6.1 The hypothalamic-pituitary-gonadal axis and sexual development

Sexual disruption occurring in wild fish is thought to occur through disruption of the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.6), which plays an important role in sexual development and reproduction in vertebrates. In fish, the HPG axis is made up of a set of tissues: the hypothalamus, pituitary, testes or ovary, as well as the liver in

females, which are connected by vascular and neuronal linkage (Ankley and Johnson, 2004). It is a dynamic but stable system under the control of hormonal feedback loops, which responds to external cues by bringing the organism to a new dynamic state, such as the initiation of a reproductive state. In this case, the hypothalamus produces GnRH, which stimulates the pituitary to produce gonadotrophic hormones, luteinising hormone (LH) and follicle stimulating hormone (FSH). Whilst FSH initiates gametogenesis, LH stimulates the production of sex steroids and causes gamete maturation, as well as ovulation or sperm release (Ankley and Johnson, 2004). Sex steroids negatively feedback on the hypothalamus to control the system by reducing the synthesis of gonadotrophins. In the ovary, LH stimulates oestrogen production (E2), from the thecal and granulosa cells, which in turn stimulates oocyte development, vitellogenin production in the liver and the initiation of female secondary sexual characteristics. These effects are mediated by oestrogen receptors, where E2 binds agonistically to initiate a transcriptional response. In males, LH increases androgen production (testosterone and 11-ketotestosterone) in the Leydig cells, which stimulate male secondary sexual characteristics and spermatogenesis, mediated through ARs. However, in the target tissue testosterone can also be converted to the more potent androgen, DHT; the major androgen in humans, the function of which is subject to increasing research in fish (Martyniuk *et al.*, 2013).

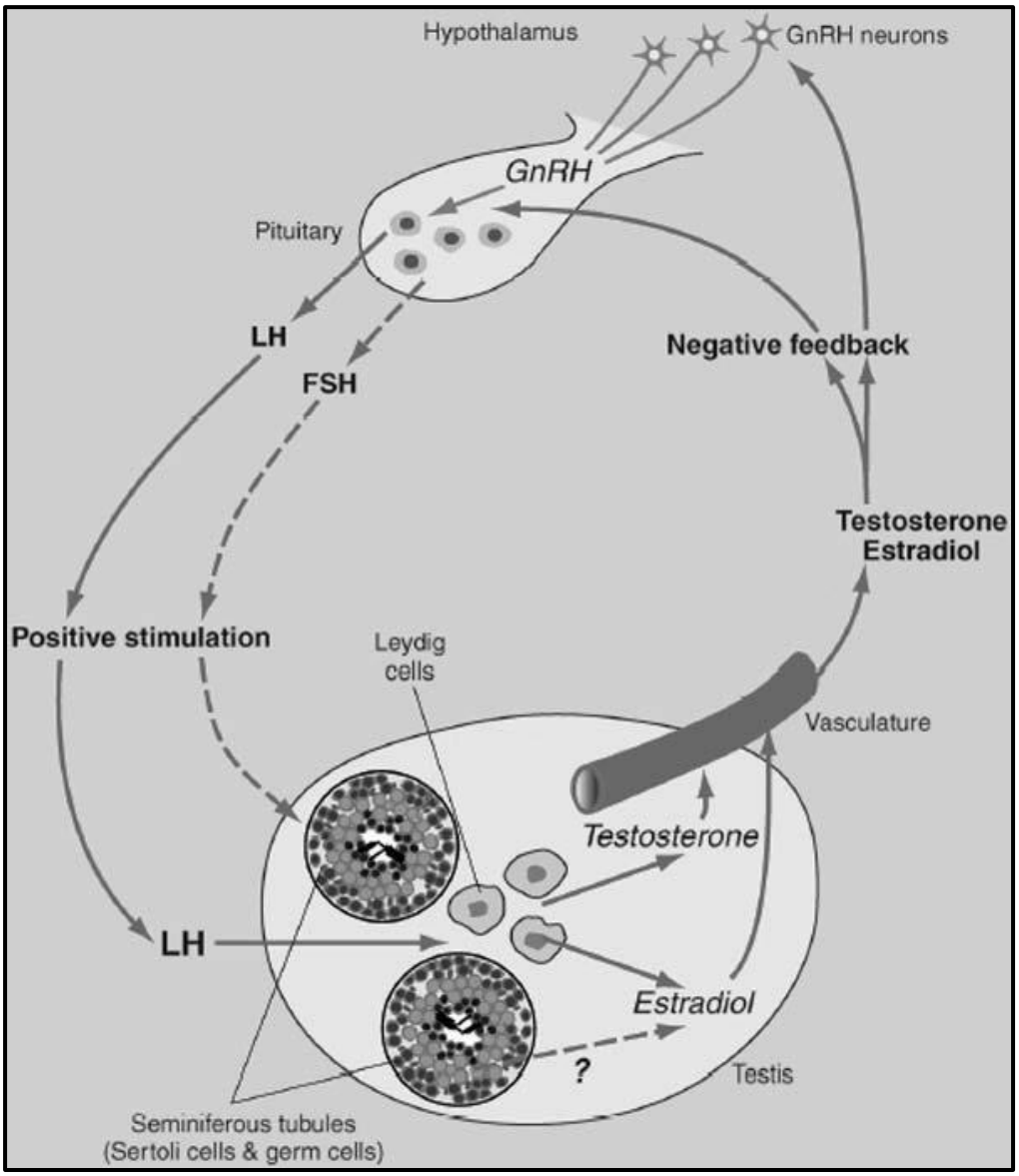


Figure 1.6 *The hormonal control of the hypothalamic-pituitary-gonadal (HPG) axis in the male teleost fish* (from Ankley and Johnson, 2004)*. GnRH = gonadotrophin releasing hormone; LH = luteinising hormone, FSH = follicle stimulating hormone.*

Sexual differentiation in fish can be triggered by genetics or environmental factors and there are a variety of mechanisms through which this is achieved (Herpin and Schartl, 2009; Devlin and Nagahama, 2002). Some species are gonochoristic, developing as males or females and remaining that way throughout their lifespans. In most gonochorists the gonad develops into a testis or ovary from an undifferentiated state, such as in Japanese medaka (*Oryzias latipes*). However, the gonad in other gonochorists can initially develop ovarian tissue, before either continuing development to become female or regressing and developing testicular tissue, such as in zebrafish

(*Danio rerio*) (Maack and Segner, 2004). Other fish naturally undergo complete sex reversal as part of their hermaphroditic life cycle with protandrous species, such as the cinnamon clownfish (*Amphiprion melanopus*), changing from males to females (Kim *et al.*, 2013). Vice versa, protogynous fish such as the three spotted wrasse (*Halichoeres trimaculatus*) start life as females (Kobayashi *et al.*, 2011). Sex change in these species can be a result of aging and increasing body size but can also be affected by social change, such as the loss of a dominant female in a protandric group. This can initiate male to female sex change in other individuals (Avise and Mank, 2009). Other hermaphroditic fish are termed simultaneous hermaphrodites, such as in mangrove killifish (*Rivulus marmoratus*) (Devlin and Nagahama, 2002; Sakakura and Noakes, 2000). These have functional ova-testes and can reproduce by alternating sperm or egg delivery or even by internal, self-fertilisation.

Sexual differentiation occurs by the differential stimulation or inhibition of components of a network of highly conserved genes downstream of a master regulatory gene. Critical to this process is the steroidogenic enzyme cytochrome P450 aromatase (*cyp19a1*), which converts androgens to oestrogens. This can be stimulated by the transcription factor *foxl2* to drive ovarian development, or inhibited by *dmrt1* to drive testicular development (Piferrer *et al.*, 2012; Piferrer and Guiguen, 2008). The endocrine system is integral to this process, where oestrogens produced by aromatase are considered as a primary cause for differentiation and maintenance of the female phenotype. In comparison, androgens are viewed as the consequence of male differentiation, since they are produced in the Leydig cells of the testes after differentiation (Piferrer *et al.*, 2012). Nonetheless, oestrogens are also produced in males and in rodent models they have been found to play an important role in normal germ cell development and fertility (Nef and Parada, 2000). Similarly, androgens in females are also thought to play a role in the development and regulation of the reproductive system, but this is not as well characterised (Margiotta-Casaluci *et al.*, 2013b; Staub and De Beer, 1997). As a result, it can be considered that the processes of normal sexual development are dependent on the balance of endogenous sex hormones, with oestrogens promoting ovarian development and androgens driving testicular development (reviewed in Devlin and Nagahama, 2002). This is demonstrated during the female to male sex change of the protogynous honeycomb grouper (*Epinephelus merra*). Here, low serum oestradiol and the degeneration of oocytes occur synchronously with increasing 11-ketotesosterone and the proliferation of spermatogenic germ cells prior to sex change (Nakamura *et al.*, 2007). Similarly, exposure of fish to oestrogens or androgens under laboratory conditions (*in vivo*) can

disrupt sexual differentiation, producing complete sex reversal or the intermediate intersex condition. Although it is clear that oestrogens and androgens can promote gonad development, exactly how the HPG axis is involved in sexual differentiation and the induction of intersex is still not fully understood (Ankley and Johnson, 2004). Indeed, alongside androgens and oestrogens, recent research suggests that anti-müllerian hormone may also be implicated. In the protandrous black porgy (*Acanthopagrus schlegeli*), low concentrations of anti-müllerian hormone were associated with the male to female sex reversal, which occurs in this species naturally during its life cycle (Wu *et al.*, 2010). Furthermore, reduced anti-müllerian hormone was also associated with the inhibition of male gonadal development in zebrafish (Schulz *et al.*, 2007).

1.6.2 Wastewater treatment works effluents and the feminisation of wild fish

The chemical causation of widespread intersex in wild fish populations remains subject to continuing research. In the UK, the presence of intersex fish was thought to be the result of feminisation of males or masculinisation of females. However, there are four major lines of evidence described by Jobling and Tyler (2008) which indicate that the feminisation of males is occurring. (1) When the populations were surveyed, the number of intersex roach was inversely proportional to the number of normal males. (2) The blood concentrations of 11-ketotestosterone and E2 in intersex fish were also similar to those of the normal males. (3) Vitellogenin induction was observed in wild male and intersex fish. (4) Effluents surveyed in the UK that were contaminating river networks tended to have oestrogenic activity, although more recently, anti-androgenic activity has also been detected (Tyler and Jobling, 2008).

Due to its continuous discharge, WWTW effluent contamination of river networks is widespread and has been strongly associated with sexual disruption in fish globally (Tetreault *et al.*, 2011; Maltret-Geraudie *et al.*, 2008; Jobling *et al.*, 2006; Woodling *et al.*, 2006; Batty and Lim, 1999). Indeed, effluent concentrations at river sites have been correlated with the incidence of intersex in fish in the UK (Jobling *et al.*, 1998). It is clear that EDCs within effluent can significantly impact reproductive health in fish and with the effects associated with feminisation, oestrogenic EDCs are likely to be significant contributors. These are oestrogen receptor agonists which can bind to oestrogen receptors to activate the transcription of oestrogen responsive genes. Indeed, oestrogenic activity is widespread in WWTW effluents globally, with a UK

survey finding activity in all 43 of its tested effluents (Johnson *et al.*, 2007b). The discharge of these effluents into the aquatic environment has also been shown to cause oestrogenic activity in rivers downstream of their outfalls (Harries *et al.*, 1997). Furthermore, *in vivo* exposures to WWTW effluents from a number of countries have been shown to induce the effects observed in the aquatic environment. Indeed, the induction of ovarian cavities occurred in roach exposed during early development in a dose dependent manner, with 100% of fish exhibiting the condition in 100% effluent (Rodgers-Gray *et al.*, 2001). In a longer term *in vivo* exposure of 3.5 years, 100% effluent produced a roach population which was phenotypically 98% female. Those suspected of being sex reversed males contributed poorly to the next generation in a competitive breeding study, although their original sex could not be confirmed (Lange *et al.*, 2011). In the same study, exposure of roach to 50% diluted WWTW effluent also found intersex induced in 21% of males. In addition, reductions in secondary sexual characteristics (Vajda *et al.*, 2011) and spermatogenesis (Kumar *et al.*, 2012) have also been observed.

Following the discovery of oestrogenic WWTW effluent, fractionation of domestic WWTW effluent samples and gas chromatography mass-spectrometry was completed to identify the chemicals responsible for this activity. This detected three steroid oestrogens including two natural oestrogens, E1 and E2 and the synthetic oestrogen and constituent of the contraceptive pill, EE2 (Desbrow *et al.*, 1998). In addition, concern was also raised about the impact of alkylphenols, which were found to be oestrogenic *in vitro* and were also common in effluents and rivers, particularly in industrial areas (White *et al.*, 1994; Jobling and Sumpter, 1993). Further investigation has also detected steroid oestrogens, alkylphenols and the plasticiser BPA in the bile of fish exposed to WWTW effluents (Pettersson *et al.*, 2006; Gibson *et al.*, 2005) and oestrogenic activity has been identified as a characteristic of a number of other chemicals found in WWTW effluent. Nonetheless, *in vitro* data suggests that it is the steroid oestrogens that are the main cause of oestrogenic activity in effluents, contributing up to 96% of the observed activity (Kinani *et al.*, 2010; Salste *et al.*, 2007; Snyder *et al.*, 2001). However, this may be different in some localities dependent on the presence of other contaminants. For example, the alkylphenols have been shown to play a greater role in industrial areas associated with their discharge (Sheahan *et al.*, 2002b).

1.6.3 The role of steroid oestrogens

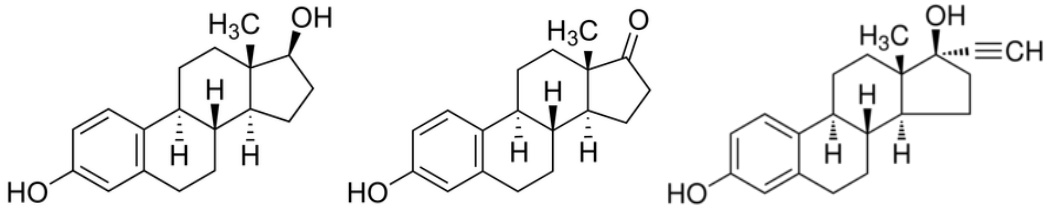


Figure 1.7 *The Steroid Oestrogens: 17β-oestradiol (E2) (left), oestrone (E1) (centre) and 17α-ethinylestradiol (EE2) (right)* (sourced from Sigma Aldrich).

The presence of the steroid oestrogens E1, E2 and EE2 (Figure 1.7) in WWTW effluent is a direct result of human excretion. Although they are excreted in their inactive conjugated forms, it is hypothesised that they are deconjugated back to their active parent compounds by *Escherichia coli* through their synthesis of the enzyme β-glucuronidase. Indeed, this occurred when activated sludge from a WWTW in Germany was spiked with oestrogen-glucuronide complexes, which proceeded to form active, free oestrogens (Ternes *et al.*, 1999). This process of activation was also demonstrated when fathead minnows were exposed to a laboratory generated effluent spiked with the the inactive oestradiol-3-glucuronide. Here, a vitellogenin induction was achieved, which supported the hypothesis of microbial deconjugation to produce active ostrogens (Panter *et al.,* 1999).

The constant release of steroid oestrogens from WWTWs has led to the widespread contamination of river networks where these chemicals are arguably pseudo-persistent (Williams *et al.*, 2009; Sumpter *et al.*, 2006). In effluents, their concentrations occur in the low ng/L, as demonstrated by a survey of 42 UK WWTW effluents which measured concentrations of <1 to 100 ng/L for E1, <1 to 22 ng/L for E2 and <1 to 3.2 ng/L for EE2 (Johnson *et al.*, 2007b). In addition, localised contamination of rivers can also result from agricultural runoff through the excretion of E1 and E2 by livestock (Matthiessen *et al.*, 2006).

Under laboratory conditions, concentrations of steroid oestrogens relevant to the environmental scenario are capable of producing the characteristics associated with sexual disruption in the wild or following effluent exposure. This was demonstrated by a study of the induction of vitellogenin in rainbow trout following exposures to E1 and E2 at 10 ng/L (Routledge *et al.*, 1998). Similarly, in a study using Japanese medaka as a

model, induction of intersex was observed with exposure to 10 ng/L E1 or E2 and 0.1 ng/L EE2. In fact, these effects have been observed following *in vivo* exposures with multiple fish species and predicted no effect concentrations (PNECs) derived from reproductive endpoints have been deduced as 6 ng/L E1, 2 ng/L E2 and 0.1 ng/L EE2 (Caldwell *et al.*, 2012). It was proposed that the steroid oestrogens could be major causal factors of endocrine disruption in wild fish; a hypothesis which was greatly strengthened by one of the UK river surveys assessing intersex in wild roach (Jobling *et al.*, 2006). This found that both the incidence and severity of intersex in fish at 45 river sites across the country were significantly correlated with the concentrations of steroid oestrogens predicted to occur at the sites. Moreover, the effects were associated with the predicted proportion of domestic effluent, from which the steroid oestrogens originate, and there was no correlation with industrial effluent. Interestingly, the correlation between the induction of vitellogenin in the sampled fish and the predicted oestrogen concentrations was not as strong, which could suggest that other factors are involved. Nonetheless, steroid oestrogen contamination is now considered to have a major impact on the reproductive health of wild fish.

Further research on the *in vivo* and *in vitro* activity of the steroid oestrogens have broadened our understanding of how they interact with fish and their potential additional impacts on wild populations. All three have a strong agonistic affinity for the oestrogen receptors and so are able to bind and induce oestrogenic responses in target tissues. The HPG axis can also be further impacted by alterations to the expression of mRNA coding for steroid receptors and genes involved in reproductive function, as observed in an exposure of fathead minnows to E2 (Filby *et al.*, 2006). In this study, increased expression of oestrogen receptor 1 (*esr1*) and a reduction in the expression of enzymes involved in androgen synthesis was observed. In addition, anti-müllerian hormone, which inhibits the development of female genitalia and favours androgen production in the steroidogenic pathway, was downregulated. In another study, EE2 also inhibited the expression of genes involved in androgen biosynthesis, including *cyp17*, *11β-hsd* and *17β-hsd*, as well as those involved in testicular development, such as *dmrt1*. In comparison, the expression of genes related to oestrogen production was increased, including *cyp19a1* (Filby *et al.*, 2007b).

It has been demonstrated that fish species differ in their sensitivity to steroid oestrogens, which is critical when interpreting results of *in vivo* studies using fish models in an environmental context. Indeed, an assessment of *in vitro* interactions with oestrogen receptor α from six fish species found that each species had different sensitivities to the three steroid oestrogens and that no one species was most sensitive

to all three. This was followed by *in vivo* exposures, which also found species differences in vitellogenin mRNA induction (Lange *et al.*, 2012b). This corresponds with the environmental scenario, where reproductive abnormalities observed in some species at a contaminated site have not been observed in others (Kavanagh *et al.*, 2004). Furthermore, the timing of exposure appears to be important in determining the developmental effect, as critical windows of exposure are evident in fish, as well as in rodents and humans. Indeed, exposure of fathead minnows at various stages of early development found a window of sensitivity following EE2 exposure at 5-10 days post hatch, where 60% of sampled males developed ovarian cavities (van Aerle *et al.*, 2002). In contrast, the induction of testicular oocytes can also occur in later life with exposure during the post spawning regrowth period in roach (Baynes *et al.*, 2012).

More extreme effects can occur in long term exposures of fish to steroid oestrogens, which incorporate large proportions of the life cycle. For example, following exposure of roach to 4 ng/L EE2, post fertilisation for up to two years, complete sex reversal was observed when a phenotypically all-female population was produced (Lange *et al.*, 2009). In addition, reproductive failure with no fertilisation was observed in breeding populations of zebrafish in a full life cycle exposure to 5 ng/L EE2. This was caused by the high incidence of non-functional testes in the male population. Whilst this decreased fecundity it did not affect behaviour, allowing reproductively compromised males to compete with normal males (Nash *et al.*, 2004). Similarly, but on a lager scale, a whole lake exposure to 5-6 ng/L EE2 over three consecutive years was completed in Canada's Experimental Lakes Area, which caused delayed spermatogenesis and intersex in male fathead minnowss, as well as altered oogenesis in females. This resulted in two years of reproductive failure, with very low recruitment and eventually caused a complete population crash (Kidd *et al.*, 2007). This has caused great concern for population level impacts on wild fish exposed to steroid oestrogens and whether this is already occurring in the wild remains an important research question.

As a result of the concerns raised by steroid oestrogens in the environment, assessments have been made to determine whether the application of improved wastewater treatment technology can reduce concentrations and mitigate the effects of steroid oestrogens on fish. In the UK's Endocrine Disruption Demonstration Program, a variety of wastewater treatment technologies, including advanced tertiary treatments, were assessed to determine their performance in removing steroid oestrogens (Butwell *et al.*, 2010). The results showed that tertiary treatment technologies could reduce concentrations to below the PNECs for E1 and E2 as well as EE2, albeit with limited additional dilution. In addition, an *in vivo* assessment of fathead minnowss found that

the effects that occurred in an effluent treated through the activated sludge process (reduced secondary sexual characteristics and vitellogenin induction) did not occur in response to the tertiary treated effluents (Filby *et al.*, 2010). In contrast, an assessment of roach exposed to some tertiary effluents still found intersex occurring, even when removal efficiency was high (Baynes *et al.*, 2012). This suggests that there could be other chemicals acting on these endpoints alone or in combination with the steroid oestrogens in the complex mixtures that make up WWTW effluent.

Research suggests that steroid oestrogens can act additively with other environmental oestrogens, such as alkylphenols and BPA (Thorpe *et al.*, 2001). Indeed, one study found that vitellogenin could be induced in fathead minnowss by a mixture of steroid oestrogens and other oestrogenic chemicals present at equipotent concentrations, which would have been too low to induce a response individually (Brian *et al.*, 2005). Crucially for this project, predictive modelling has also proposed a role for anti-androgenic contaminants in the causation of endocrine disruption in fish, which will be further discussed (Jobling *et al.*, 2009). Nonetheless, whilst they may be part of a more complex picture, steroid oestrogens still appear to be the major causal components of endocrine disruption in fish. As a result of this, in 2012, E2 and EE2 became the first pharmaceuticals to be considered for regulation under the European Water Framework Directive with their addition to a watch list by the European Commission (Owen and Jobling, 2012).

1.6.4 Alkylphenols

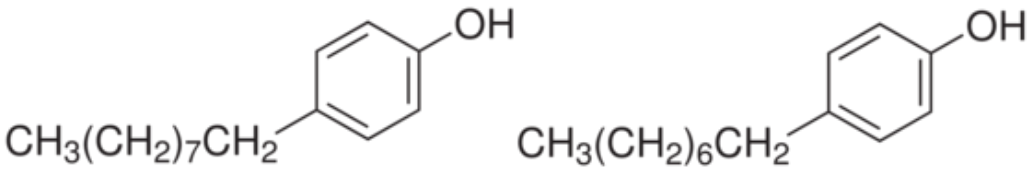


Figure 1.8 *The alkylphenols: 4-nonylphenol (NP) (left) and octyphenol (OP) (right)* (sourced from Sigma-Aldrich).

Alkylphenolic chemicals are also thought to have contributed to feminisation of wild fish alongside steroid oestrogens and in some highly contaminated areas they have been more significant contributors to oestrogenic activity (Sheahan *et al.*, 2002b). These include NP, OP (Figure 1.8), their mono to tri-ethoxylate derivatives (NPE1, NPE2 and NPE3) and carboxylate derivatives, which were found to be oestrogenic *in vitro* and

widespread in the aquatic environment. They are the persistent degradation products of alkylphenol polyethoxylates, which are used as non-ionic surfactants in a variety of industrial applications, including textiles, paints, pesticides and plastics, as well as industrial and household cleaning products (Ying *et al.*, 2002b; White *et al.*, 1994). Like the steroid oestrogens, they enter the aquatic environment through WWTW effluents, but they also have a direct input from their industrial use (Hale *et al.*, 2000). Due to their high LogKow, they partition effectively into sediments following wastewater treatment, which in conjunction with the water concentrations provides an extensive reservoir in the environment (Ying *et al.*, 2002b). Indeed, environmental concentrations have been observed which are much higher than the steroid oestrogens. In the UK, between 1993 and 1994, up to 330 µg/L of NP was detected in WWTW effluent discharging to the River Aire catchment. This contained discharge from the textiles industry and further assessment detected up to 180 µg/L in the river itself (Blackburn and Waldock, 1995). Similar concentrations have been observed worldwide but tend to vary extensively between WWTWs and between countries dependent on the regulation of alkylphenols, which is lacking outside of Europe (Ying *et al.*, 2002b).

The *in vitro* oestrogenic potency of the alkylphenols has been tested in multiple assays and was found to be over 1,000 times lower than E2 (Metcalfe *et al.*, 2001; White *et al.*, 1994; Jobling and Sumpter, 1993). Furthermore, their association with the oestrogen receptor in exerting oestrogenic activity has been confirmed in co-exposures with the oestrogen receptor antagonist tamoxifen, which reduced NP induced vitellogenin induction by rainbow trout hepatocyte cell lines (Jobling and Sumpter, 1993). *In vivo* assessments of NP and OP have also demonstrated their capacity to induce sexual disruption in fish species, including intersex and vitellogenin induction, as well as a potential to bioaccumulate in tissues (Soares *et al.*, 2008). Similarly to *in vitro* data, alkylphenols also require much greater concentrations than the steroid oestrogens to exert affects *in vivo*. For example, in one study of the effects of NP in rainbow trout, vitellogenin induction occurred at 20.3 µg/L (Jobling *et al.*, 1996), whilst another study observed induction at 150 µg/L in exposures to NP and OP. In this case, OP produced a greater effect than NP based on vitellogenin measurements taken before and after exposure, which agrees with the higher oestrogenic activity of OP *in vitro* (Pedersen *et al.*, 1999). However, a study of intersex induction in an early life exposure of Japanese medaka found their lowest effect concentrations (LOECs) to be similar; at 11.6 and 11.4 µg/L for NP and OP, respectively (Seki *et al.*, 2003). Furthermore, whilst *in vitro* effects on trout hepatocytes showed that some nonylphenol ethoxylate derivatives have equal or higher oestrogenic activity, they responded very weakly *in vivo* with

intersex induction at 100 µg/L, suggesting that NP and OP are a greater cause for concern (Metcalfe *et al.*, 2001).

Although the potency of the alkylphenols is lower than the steroid oestrogens, their concentrations are much higher and have reached levels in effluents and rivers where *in vivo* effects have been observed (Blackburn and Waldock, 1995). As a result, alkylphenols, and in particular NP and its ethoxylates due to its higher use, have been associated with oestrogenic activity and adverse effects on fish. Indeed, high vitellogenin responses and lower gonadosomatic indices (GSI) of caged rainbow trout on the River Aire was thought to be caused by contamination by alkylphenols. These occurred at concentrations sufficient to retard testicular growth *in vivo* due to the input of highly contaminated trade waste from the textiles industry to a WWTW discharging to the river (Sheahan *et al.*, 2002b; Harries *et al.*, 1997). However, discharge regulations were tightened on the River Aire, leading to improvements in the wastewater treatment process and a reduction in the output volume of effluent. Consequently, NP concentrations downstream of the discharge decreased from 180 µg/L to 2 µg/L and improvements in GSI and reductions in vitellogenin in caged rainbow trout were also observed (Sheahan *et al.*, 2002a). This strengthened the case for the environmental, endocrine disrupting impact of NP in wild fish.

In part because of their endocrine activity, but primarily based of their acute toxicity, the EU passed legislation restricting the uses of non-ionic surfactants based on NP as part of the Water Framework Directive (Sumpter and Jobling, 2013). There is also increased monitoring in Japan and Canada, whilst the US EPA published guidelines on NP and its ethoxylates in the environment (Soares *et al.*, 2008). More recently, the US EPA has also begun to support voluntary phase out and regulatory action to manage the risk posed by these chemicals (U.S. Environmental Protection Agency, 2010). Nonetheless, NP is still detectable in the aquatic environment in Europe, but at much lower concentrations than prior to regulation. Although its ability to induce significant impacts alone has probably reduced, it still may interact additively in a mixture scenario with other oestrogens. It may also function through other mechanisms and has been identified as anti-androgenic *in vitro* in the ASC1 yeast two hybrid system (Lee *et al.*, 2003). In addition, there are still no restrictions on use in a number of countries outside of Europe, with Australia, South America, China and India still using large quantities.

1.6.5 Other oestrogenic contaminants

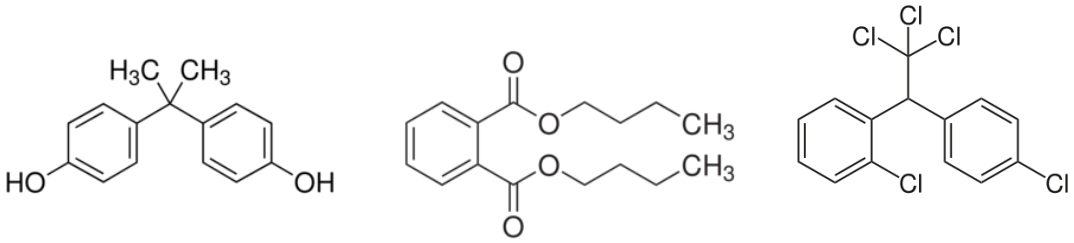


Figure 1.9 *Additional examples of weakly oestrogenic environmental contaminants. Bisphenol A (left), dibutyl phthalate (centre) and o,p'-dichlorodiphenyltrichloroethane (DDT) (right)* (sourced from Sigma Aldrich and Wikimedia Commons).

Other low potency oestrogenic contaminants have also been identified in the aquatic environment, including plasticisers such as BPA and the phthalate esters, pesticides, parabens and PCBs (examples shown in Figure 1.9) (Turner and Sharpe, 1997). Their *in vitro* activity ranges widely, with the potencies of plasticisers and pesticides ranging from 5,000 to 2.5 million times lower than E2 (Hurst and Sheahan, 2003; Harris *et al.*, 1997). Indeed, vitellogenin induction caused by plasticisers required high concentrations that exceeded environmental concentrations (Oehlmann *et al.*, 2009; Sohoni *et al.*, 2001). However, some have been shown to act on multiple receptors, including antagonistically with the AR (Sohoni and Sumpter, 1998). This suggests that other mechanisms of action may be more relevant in causing the effects of phthalates at lower concentrations. In the case of BPA, a number of studies have observed effects at environmentally relevant concentrations, including two multi-generational studies, which found altered spermatogenesis and reduced hatching success in F1 fathead minnowss at 16 µg/L (Staples *et al.*, 2011; Mandich *et al.*, 2007; Kwak *et al.*, 2001; Sohoni *et al.*, 2001). Intersex was also induced, with a LOEC of 10 µg/L (Metcalfe *et al.*, 2001), in comparison to the highest detected environmental concentration of 21 µg/L in surface water from the Netherlands (Belfroid *et al.*, 2002). This suggests that BPA could affect fish reproductive health at its higher environmental concentrations. In contrast, phthalate esters have not been detected in the environment at concentrations where intersex could be induced in laboratory studies (Norman *et al.*, 2007). However, there is some evidence of adverse effects on sperm at lower concentrations, although this may be mediated through the PPARs (Oehlmann *et al.*, 2009). This suggests that phthalates may not be significant contributors to sexual disruption observed in wild fish by an oestrogenic mechanism.

Pesticides are similar in this respect, where the concentrations occurring in agricultural headwaters in the UK were only found to make a small contribution to the total oestrogenic activity observed (Hurst and Sheahan, 2003). In addition, concentrations of the persistent oestrogenic pesticide DDT have dramatically reduced in the western world, limiting its impact in the aquatic environment in recent years. Nonetheless, in parts of the world where DDT is still in use, biomonitoring programs are continuing in areas where DDT spraying still occurs (Brink *et al.*, 2012) and assessments of the concentrations reached in the Pearl River Delta in China, suggest that there is risk of adverse effects in fish (Guo *et al.*, 2009). However, it is important to note that DDT represents a mixture of isomers and whilst o,*p'*-DDT is oestrogenic, it has also been shown to have anti-androgenic activity, as has the DDT metabolite *p,p'*-DDE (Sohoni and Sumpter, 1998; Kelce *et al.*, 1995). Indeed, in an environmental scenario at Lake Apopka, Florida, the reduced phallus size in alligators is thought to be a result of anti-androgenic demasculinisation due to the accumulation of *p,p'*-DDE (Guillette Jr. *et al.*, 1996).

Interestingly, equine oestrogens have been also detected in the bile of fish exposed to WWTW effluents, alongside the steroid oestrogens and nonylphenol (Gibson *et al.*, 2005). Further assessment identified equilenin and its metabolite 17β-dihydroequilenin, which was first detected in fish bile, in sewage influents at sub ng/L to low ng/L concentrations (Tyler *et al.*, 2009). Whilst their origins are equine, they are extracted from horse urine for use in pharmaceuticals for hormone replacement therapy, which seems a likely origin for the compounds in influent and effluent. In addition, their low concentrations suggested that they were being bioconcentrated in fish and *in vivo* testing found that they could induce vitellogenin in rainbow trout at 0.6 and 4.2 ng/L, respectively. Consequently, they are still considered highly likely that they could contribute towards feminisation in wild fish (Tyler *et al.*, 2009).

1.6.6 Mixture effects of oestrogenic chemicals

In the last decade there has been an increasing appreciation of the importance of testing chemicals in combination in environmentally relevant exposure scenarios. Indeed, both humans and wildlife are exposed to mixtures of environmental contaminants simultaneously. As a result, it has been argued that the use of single chemical studies could lead to the underestimation of the risk that a chemical could pose in a real world scenario and that a cumulative risk assessment approach would be more applicable (Kortenkamp and Faust, 2010). This has led to an increase in the

importance of understanding mixture effects from both research and regulatory perspectives and the growth of the field of mixtures toxicology (WHO/UNEP, 2013; Kortenkamp, 2007).

When combined, mixtures of chemicals can act additively according to the concept of dose addition, which has been well documented in interpreting the effects of mixtures of endocrine disrupting chemicals. This states that one chemical can be replaced totally or in part by an equal fraction of an equieffective concentration of another without diminishing the overall combined effect (Kortenkamp, 2007). Based on this assumption, the mixture effect of a defined set of chemicals known to impact an endpoint can be calculated from the dose responses of single chemicals. However, mixtures may also act synergistically (causing effects greater than if they acted additively) or antagonistically (causing effects below the expected additivity) (Kortenkamp, 2007). Additive action has been demonstrated for a set of environmental oestrogens, which were described as producing a "something from nothing" effect. Here, a group of xenoestrogenic chemicals, including parabens, bisphenol A (BPA) and genistein, were combined at concentrations below their individual NOECs but still produced a significant effect *in vitro* (Silva *et al.*, 2002). Further *in vitro* study also found that the combined effect of 11 xenoestrogens at concentrations below their individual NOECs dramatically enhanced the action of 17β-oestradiol (E2) (Rajapakse *et al.*, 2002). Consequently, whilst some oestrogens in the environment may not be present at sufficient concentrations to act alone, they may act in combination to cause a significant biological effect.

This concept was eventually demonstrated *in vivo* using fathead minnows under singular and combined exposures to five oestrogenic chemicals (E2, EE2, BPA, nonylphenol and octylphenol) using the endpoint of vitellogenin induction. In this case, when the individual chemicals were dosed at concentrations equal to a fifth of their EC50 values, they failed to induce a significant vitellogenin response. However, a significant response was induced when they were combined, which was consistent with the predictions made based on dose addition (Brian *et al.*, 2005). Further study of these five oestrogenic chemicals showed that they also affected fitness and fecundity, with secondary sexual characteristics and egg production reduced in mixture treatments (Brian *et al.*, 2007). This is shown below in Figure 1.10.

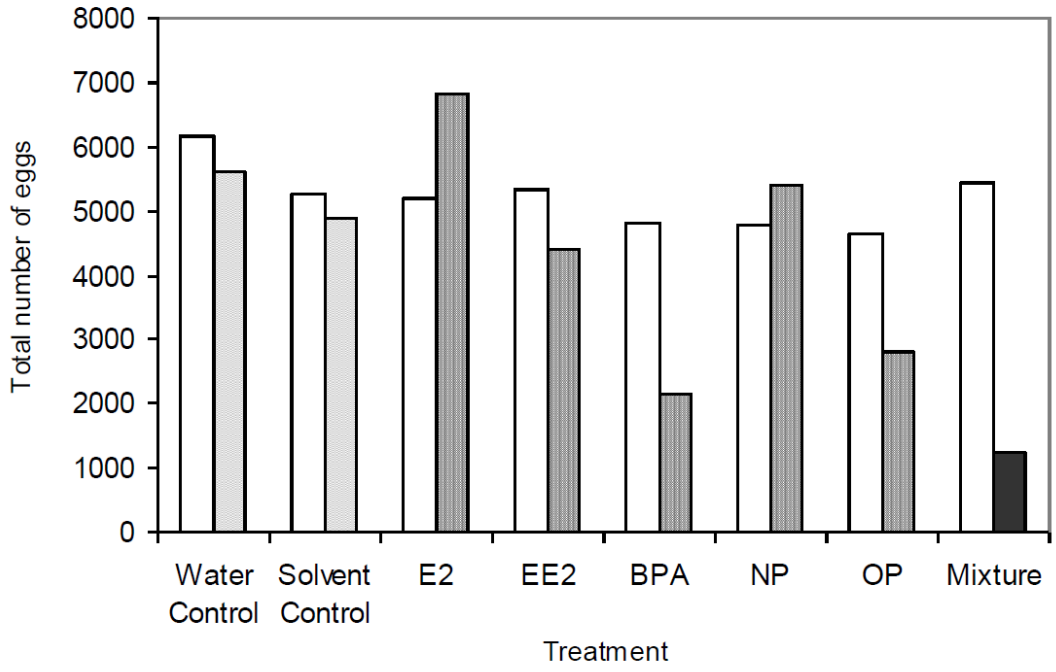


Figure 1.10 *The combined effects of five oestrogenic chemicals on egg production in fathead minnows, with the pre and post exposure spawning period represented by clear and shaded bars respectively. The concentrations were 70 ng/L E2, 1.5 ng/L EE2, 500 µg/L bisphenol A (BPA), 5 µg/L nonylphenol (NP) and 5 µg/L octylphenol (OP). BPA caused a significant reduction in egg count alone. However, the magnitude of the difference in egg production between the pre and post-spawning period indicate a more pronounced response in the mixture* (from Brian *et al.*, 2007)**.**

1.7 Sexual disruption in male fish: demasculinisation and a role for anti-androgens

Although oestrogenic activity has been strongly associated with sexual disruption of fish in the aquatic environment, it is not the only mechanism of endocrine disruption through which these effects can be induced. In the UK, the number of intersex roach was found to be inversely proportional to the number of normal males, suggesting that this is a change of male fish to an intersex state in a “feminisation” process. However, the same effects can also occur through “demasculinisation,” as a result of exposure to anti-androgens. These are substances which can reduce the concentrations of circulating androgens and/or can interrupt their activity at the target site, disrupting the function of the HPG axis. In recent years, interest has been growing regarding the

increasing number of environmental chemicals being identified as anti-androgens and the presence of anti-androgenic activity in environmental samples. Indeed, an estimated 8% of all known chemicals are thought to be anti-androgenic, a number which based on the European chemical market could run into thousands (Kortenkamp and Faust, 2010; Vinggaard *et al.*, 2008). However, the chemicals responsible for this activity in the environment remain unknown to a large extent and their effect on wild fish populations is still under research. Nonetheless, androgens including testosterone, 11-ketotestosterone and possibly DHT play a crucial role in development and reproduction in fish (Martyniuk *et al.*, 2013). These effects are mediated through the ARs, of which two isoforms have been identified in some teleost species (Sone *et al.*, 2005; Sperry and Thomas, 1999). Androgens are produced by Leydig cells in the testes, although there is evidence for other sources in fish. They are subject to seasonal changes, reaching their highest concentrations during spawning. As well as promoting male gonad development, androgens also promote male sexual behaviour, secondary sexual characteristics and somatic growth (Borg, 1994). As a result, they can determine the reproductive strategy of a male (Brantley *et al.*, 1993). They are also present in females as precursors to oestrogens and are thought to play an important role in the development and regulation of the reproductive system (Staub and De Beer, 1997). In addition, a survey of microarray and proteomic studies shows that there are a number of additional pathways regulated by androgens, including lipid metabolism and oxidation, immune response, protein metabolism and muscle proliferation (Martyniuk and Denslow, 2012). As a result, the potential for disruption to endogenous androgens in wild fish is a cause for concern.

1.7.1 The modes of action of anti-androgens

Traditionally, anti-androgens have been considered as substances which can block androgen action at the receptor in an antagonistic fashion. However, it is now clear that anti-androgenic activity has a much wider scope than this. Indeed, as well as interactions at the receptor, some anti-androgens can act by testosterone synthesis inhibition or catabolism of androgen precursors of testosterone itself. There is also evidence of mixed mechanism anti-androgens, as well as anti-androgenic interactions between the aryl hydrocarbon receptor (AhR) and the AR (Crago and Klaper, 2012; Wilson *et al.*, 2008; Kizu *et al.*, 2003a). However, whilst these mechanisms are well studied in mammalian models and in *in vitro* assays, there are less available data on mechanisms of action in fish that could cause a demasculinising response.

1.7.1.1 Interactions at the androgen receptor

Many anti-androgens act as receptor antagonists, which are considered to be ligands that bind to a receptor without inducing a biological effect. Consequently, evidence for AR antagonism tends to originate from androgen responsive *in vitro* cell lines transfected with an AR. However, these may not necessarily correspond to activity *in vivo*. Anti-androgenic AR antagonists can bind competitively to the receptor site to block the action of androgens in the target cell, inhibiting androgen induced transcriptional activation. This is generally considered to occur by preventing the binding of the receptor to the androgen response element of the DNA, a mechanism which has been demonstrated for anti-androgens including the DDT metabolite *p,p'*-DDE and the pesticides vinclozolin and methoxychlor (Kelce *et al.*, 1998; Kelce and Wilson, 1997). However, there are relatively few anti-androgens which have purely antagonistic activity in their receptor binding (Singh *et al.*, 2000), such as the human pharmaceuticals flutamide, bicalutamide and nilutamide. Most anti-androgens appear to have a mixture of agonistic and antagonistic activity, meaning that in the absence of an androgen they are weakly androgenic at higher concentrations. As a result they could also be considered as partial AR agonists, causing an anti-androgenic response by blocking the binding of more potent endogenous androgens (Nguyen *et al.*, 2007). In addition, there is also evidence for non-competitive antagonism of the AR by NP and BPA. In this case, whilst they could interfere with AR translocation to the nucleus and inhibit AR mediated transcription, they only partially inhibited the binding of DHT, suggesting that they could also be binding to an allosteric site (Lee *et al.*, 2003). This work also highlighted an inhibitory effect of these chemicals on the AR interaction with enzyme co-activators, which would normally enhance the androgenic response. Indeed, there is also *in vitro* evidence that anti-androgen action at the AR can be enhanced by an interaction with co-repressors. These are transcription factor proteins which can inhibit the ability of the receptor to activate gene expression. In one *in vitro* study, the co-infection of an anti-androgen and nuclear receptor co-repressor 1 caused a significantly greater decrease in AR activity than during exposure to the anti-androgen alone (Berrevoets *et al.*, 2002). Indeed, there is some evidence that the *in vitro* anti-androgenic effects of some PAHs may be mediated through co-repressors. In this case, it was found that some PAHs which were shown to induce an anti-androgenic response in the LNCaP cell line did not bind to the AR itself. However, they were AhR agonists and it was suggested that the anti-androgenic effect was caused by

AhR activation of the transcription factor, activator protein 1 (AP-1), which interacted with the AR to inhibit its binding to the androgen response element (Kizu *et al.*, 2003a).

Blocking the function of androgens at the AR causes deficient androgen action at a target site and can have significant impacts on development, which are similar to those induced by oestrogenic chemicals following exposure during a critical window. Indeed, maternal exposure of rats to flutamide caused reproductive malformations to occur in the male offspring, including cryptorchidism, hypospadias and reductions in ano-genital distance (Welsh *et al.*, 2008). The blockade of androgens has also been observed in fish where exposure to AR antagonists mitigates the effects of androgen exposure. For example, the development of male secondary sexual characteristics induced in female fathead minnowss by exposure to the androgenic chemical trenbolone was blocked by flutamide (Ankley *et al.*, 2004). Furthermore, the induction of the androgen dependent protein spiggin in female stickleback (*Gasterosteus aculeatus*) by DHT was reduced by exposure to anti-androgens such as flutamide, vinclozolin, the herbicide linuron and the insecticide fentitrothion (Jolly *et al.*, 2009). In exposures to AR antagonists alone, demasculinisation effects also occur in fish that show similarities to those observed in oestrogen exposures. For example, inhibited gonopodium length, reduced male colouration and sperm count, as well as suppression of courtship behaviour have all been identified in male guppies (*Poecilia reticulata*) fed *p,p'*-DDE, vinclozolin or flutamide (Bayley *et al.*, 2002; Baatrup and Junge, 2001). However, the concentrations used in these studies exceeded environmental relevance for these chemicals. Nonetheless, the anti-androgenic effects of flutamide have been observed in fish at concentrations equivalent to the activity detected in the aquatic environment by *in vitro* assays. These include reductions in secondary sexual characteristics (939 µg/L) and fecundity (651 µg/L) in fathead minnowss (Jensen *et al.*, 2004; Panter *et al.*, 2004), reduced courtship (100-500 µg/L) and nest building (100 µg/L) behaviour in stickleback (Sebire *et al.*, 2008) and intersex induction in Japanese medaka with a LOEC of 202 µg/L (León *et al.*, 2007; Kang *et al.*, 2006).

AR antagonists can produce an internal environment that favours the action of oestrogen due to feedback mechanisms within the endocrine system. By blocking the AR in the brain, the negative feedback of testosterone is inhibited and as a result, increased concentrations of gonadotropins are produced, which can positively stimulate sex steroid production. Whilst this increases androgen concentrations, they remain ineffective due to the anti-androgenic blockade of the AR. However, circulating E2 concentrations also increase, which have been linked to adverse “feminisation” effects, such as gynocomastia, in human male patients treated with anti-androgenic

pharmaceuticals (Staiman and Lowe, 1997). Indeed, the steroid profiles indicative of this mechanism have also been observed in both fish and rats in response to anti-androgen exposure (Jensen *et al.*, 2004; O'Connor *et al.*, 2002). Furthermore, flutamide has also been shown to upregulate genes coding for steroidogenic enzymes, as well as oestrogen receptors β and γ, in fathead minnowss (Filby *et al.*, 2007b). It is therefore highly plausible that this mechanism could also play a role in demasculinisation effects in fish. Interestingly, this study also showed some commonalities in the mechanisms of action of flutamide and EE2. These both increased expression of genes coding for oestrogen-producing enzymes (*cyp19a* and *cyp19b*) and decreased expression of genes involved in testis differentiation, such as anti-müllerian hormone and *dmrt1*.

It is important to note that species differences exist in the responses of fish to AR antagonists identified in mammals due to subtle differences in AR's between species. Indeed, mammalian AR antagonists including flutamide, vinclozolin and procymidone did not bind to goldfish (*Carassius auratus*) or rainbow trout ARs in cytosolic fractions (Wells and Van Der Kraak, 2000). However, a study of fathead minnowss and rainbow trout AR transfected into cell lines found that flutamide, vinclozolin and its metabolites bound to both receptors (Wilson *et al.*, 2007). Indeed, these chemicals have both been found to have anti-androgenic activity in fathead minnowss *in vivo* (Martinović *et al.*, 2008). Whilst this demonstrates that differences in test methods may produce different results, it also shows that different fish species can also differ in their response to anti-androgens. Indeed, cyproterone acetate, methoxychlor and *p,p'*-DDE were found to bind to the AR in goldfish but not rainbow trout, with the binding affinity of *p,p'*-DDE being similar to its affinity to mammalian receptors (Wells and Van Der Kraak, 2000). This clearly demonstrates the need to better understand which anti-androgens could impact fish health and the importance of testing these chemicals in relevant fish species.

1.7.1.2 Reducing concentrations of circulating androgens

As well as interactions with the AR, some anti-androgens can induce effects by reducing the concentrations of available circulating androgens. Mechanisms include the reduction of androgen synthesis or an increase in their catabolism through interactions with enzyme function or gene expression, which have been assessed in whole animals through molecular techniques and gene expression profiling. For example, in mammalian models, phthalate esters are generally considered to be

testosterone synthesis inhibitors. Indeed, DEHP and DBP can both reduce foetal testosterone production and downregulate genes coding for steroidogenic enzymes, such as *cyp17* and *cyp11,* and those involved in cholesterol transport, such as *StAR* (steroidogenic acute regulatory protein) (Figure 1.11). In addition, a reduction in the expression of *insl3*, a hormone secreted by the Leydig cells that promotes testicular descent, has also been linked with cryptorchidism (Nef and Parada, 2000). This genetic effect has not been observed with AR antagonists, such as vinclozolin, reflecting the higher incidence of cryptorchidism in mammalian phthalate exposures (Wilson *et al.*, 2008; Borch *et al.*, 2006). These effects are thought to be linked to downregulation of transcription factors such as PPARs and steroidogenic factor 1, which regulate these enzymes (Borch *et al.*, 2006). Indeed, the interactions of anti-androgens with the PPARs have also been linked to disruption of enzymes involved in steroid catabolism and could affect circulating androgens though their production and degradation (Fan *et al.*, 2004).

The effects on fish are not as well researched. There is evidence that the 5α-reductase enzyme, which converts testosterone to DHT in target tissues, can be inhibited by DEHP and DBP in *in vitro* assays using carp testicular tissue (Thibaut and Porte, 2004). However, *in vivo,* no effects on steroidogenic enzymes have been observed in stickleback exposed to DBP (Aoki *et al.*, 2011) nor in zebrafish and fathead minnowss exposed to DEHP (Crago and Klaper, 2012; Uren-Webster *et al.*, 2010). Nonetheless, in the DEHP exposure of fathead minnows, an increase in enzymes involved in steroid catabolism in the liver and testes occurred, which was thought to contribute to the significant reduction in plasma testosterone observed when DEHP was combined in an exposure with the herbicide linuron (Crago and Klaper, 2012). However, the exposure of stickleback to DBP did show anti-androgenic effects, in terms of a reduction in spiggin production in males at concentrations near the highest environmental concentrations (Aoki *et al.*, 2011). Consequently, the mechanisms of anti-androgenic action of phthalates in fish remain unclear and may differ from those observed in mammals.

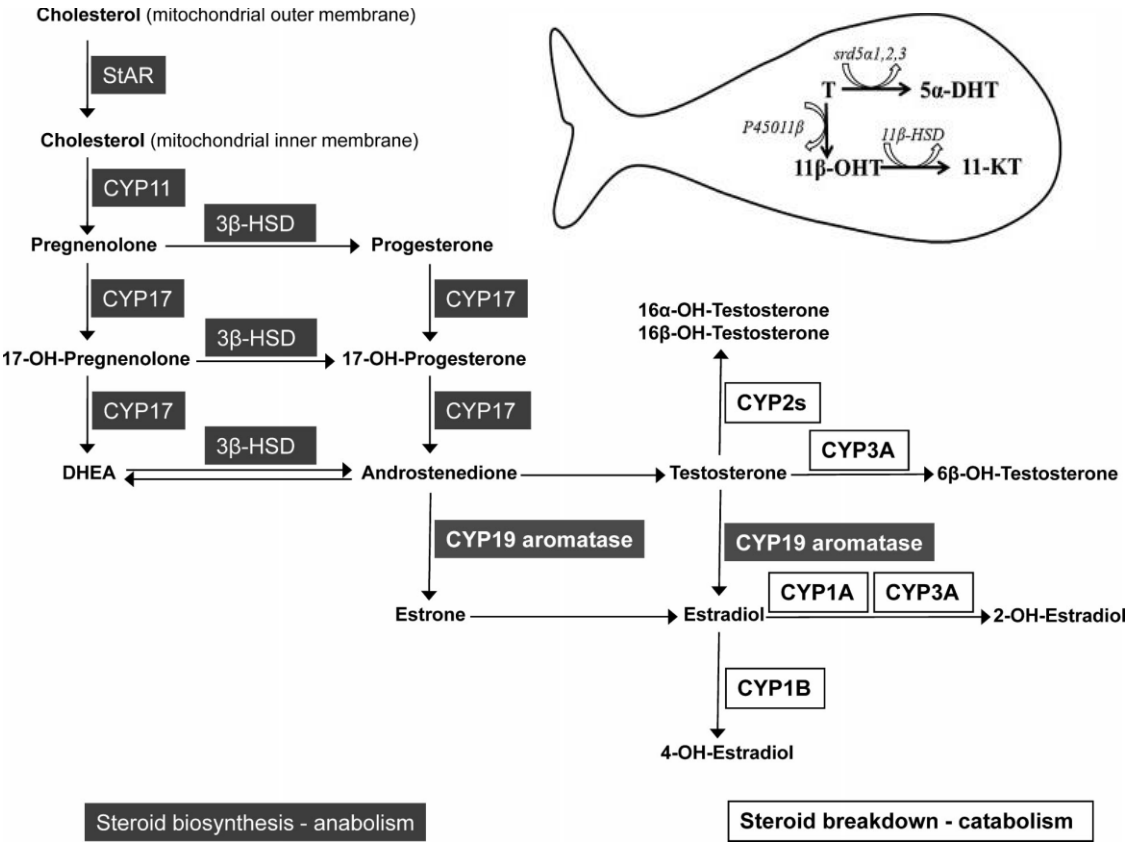


Figure 1.11 *The steriodogenesis pathway* (from Celander *et al.*, 2011) *expanded to include a simplified depiction of the conversion of testosterone to other functional androgens in fish (top-right)* (from Martyniuk *et al.*, 2013). *T = testosterone; 5α-DHT = 5α-dihydrotestosterone; 11β-OHT = 11β-hydroxytestosterone; 11-KT = 11-ketotestosterone. Srb5α1,2,3 = 5α-reductase; P45011β = cytochrome P450 11β-hydroxylase (CYP11β); 11β-HSD: 11β -hydroxysteroid dehydrogenase.*

1.7.1.3 Mixed mechanisms

Many chemicals do not act solely via a single mechanism of action and instead work through multiple mechanisms to induce their effects. For example, the fungicide prochloraz is an AR antagonist but has also been observed to reduce plasma testosterone in fathead minnows, potentially via inhibition of the activity of the steroidogenic enzyme *cyp17* (Ankley *et al.*, 2009). In addition, many AR antagonists can also act as 5α-reductase inhibitors in mammalian cell lines, including *p,p'*-DDE, vinclozolin, prochloraz and even flutamide at high concentrations (Lo *et al.*, 2007). This mechanism of action has only been recently explored in fish and there is evidence that 5α-reductase inhibitors can cause adverse effects in fish, with exposure to the pharmaceutical dutasteride causing reduced fecundity in fathead minnows (Margiotta-

Casaluci *et al.*, 2013b). Moreover, interactions can occur with other receptors and hormone types, with a number of pesticides acting as both AR antagonists and oestrogen receptor agonists (Kojima *et al.*, 2004), whilst effects of cyproterone acetate, vinclozolin and *p,p'*-DDE on thyroid parameters have also been observed in male rats (O'Connor *et al.*, 2002).

1.7.1.4 Mixture effects of anti-androgens

Anti-androgenic environmental contaminants have also been shown to act additively *in vitro* in mixture scenarios, even when a variety of chemical structures from 17 compunds were involved (Ermler *et al.*, 2011). Similar results have also been achieved *in vivo* in F1 rats where maternal exposure to a mixture of three AR antagonists (flutamide, vinclozolin and procymidone) exacerbated demasculinisation effects. These included reductions in anogenital distance and nipple retention, which occurred to a much greater extent than in single chemical exposures (Hass *et al.*, 2007). More recently, additive effects have also been confirmed in fish, where a mixture of four AR antagonists (vinclozolin, fenitrothion, flutamide and linuron) caused a significantly greater inhibition of 5α-dihydrotestosterone induced spiggin in female stickleback than they could alone. Again, this agreed well with the concept of dose addition (Pottinger *et al.*, 2013).

Interestingly, mixture studies have also shown that chemicals with similar mechanisms of action can act additively on common endpoints. In these cases, an alternative concept of independent action can be used as well as dose addition to predict additive effects. This uses a statistical concept of independent events, where individual components produce effects independent of the other agents within the mixture (Bliss, 1939). However, this can still produce similar predictions to those of dose addition (Hadrup *et al.*, 2013). One example of a study using these concepts was a maternal exposure of rats, which found that exposure to DBP (a testosterone synthesis inhibitor) and procymidone an (AR antagonist), still had dose additive effects on the same endpoints (Hotchkiss *et al.*, 2010). A second example is another maternal exposure of rats to a mixture of AR antagonists (vinclozolin and prochloraz), a testosterone synthesis inhibitor (di[2-ethylhexyl] phthalate) and a 5α-reductase inhibitor (finasteride) to assess impacts on sexual development. Additive effects were observed for increasing nipple retention and reduced prostate weight and ano-genital distance in rats, consistent with dose addition. However, synergistic effects were also observed for

genital malformations, where the mixture caused a greater response than those predicted based on dose addition and independent action (Christiansen *et al.*, 2009).

1.7.2 Anti-androgenic activity in the environment

The use of *in vitro* bioassays containing ARs has been used globally to detect anti-androgenic activity in a similar fashion to those used for assessing oestrogenic contamination. The resultant activity has tended to be reported as the flutamide equivalent concentration (FluEQ), which is widely used as a positive control in these assays. Nonetheless, anti-androgenic activity in the environment is not as well documented as oestrogenic activity. In the UK, the survey of 43 effluents completed by the Environment Agency to evaluate oestrogenic contamination also discovered anti-androgenic activity in all samples ranging from 21.3 to 1,231 µg/L FluEQ (Johnson *et al.*, 2007b). Similarly, anti-androgenic activity has also been observed in tertiary treated WWTW effluents in Australia, which could suggest that the causal chemicals were difficult to remove in sufficient concentrations (Kumar *et al.*, 2012). However, a study of domestic wastewater and greywater in China found a high rate of removal with membrane bioreactors, but due to the high anti-androgenic activity detected pre-treatment (3.1 and 1.1 mg/L FluEQ respectively), the activity in effluent was still detectable at 69 and 54 µg/L FluEQ (Ma *et al.*, 2013). Combined, these data suggest that the sources of anti-androgens are likely to be domestic and potentially consist of a low number of high volume chemicals or a high number of low volume use chemicals. However, high concentrations of anti-androgenic activity (up to 8000 µg/L FluEQ) have also been detected in offshore produced water discharges from oil platforms in the North Sea (Thomas *et al.*, 2009; Tollefsen *et al.*, 2007). Here, effect directed analysis identified a set of polycyclic aromatic hydrocarbons and napthenic acids as AR antagonists, which suggests that there could also be industrial and even natural sources for anti-androgens, since napthenic acids occur naturally in crude oil and oil sands (Thomas *et al.*, 2009).

There are some reports of anti-androgenic activity occurring in river networks. At sites on the Pearl River system in China, anti-androgenic activity alongside oestrogenic activity was detected in all samples with up to 935 µg/L FluEQ. In contrast, androgenic and anti-oestrogenic activity was found in only 41% and 29% of samples, respectively (Zhao *et al.*, 2011). In the UK, similar concentrations were detected with a maximum of 1,070.5 µg/L FluEQ occurring 0.1 km downstream of a WWTW effluent outfall prior to its treatment upgrade (Grover *et al.*, 2011). Furthermore, anti-androgenic activity in the

River Lambro in Italy was detected in both surface water and sediment, although following fractionation and *in vitro* screening, the chemicals causing the activity remained unknown (Urbatzka *et al.*, 2007). Similar findings occurred in France, where the anti-androgenic activity detected in sediment from the Rhonelle River could not be explained by the presence of BPA, alkylphenols or a set of pesticides which were analysed (Kinani *et al.*, 2010). However, an alternative method for detecting anti-androgens through the analysis of fish bile, following exposure of rainbow trout to an anti-androgenic effluent, identified a novel set of anti-androgens. These accounted for around 61% of the activity detected, with 51% made up by the biocides triclosan and chlorophene (Rostkowski *et al.*, 2011). Nonetheless, it is clear that the causes of anti-androgenic activity still remain largely unknown and it should also be considered that activity based on AR binding may not be representative of all environmental anti-androgens since some can exert their effects through alternative mechanisms.

Based on *in vivo* exposures of fish to flutamide alone, the anti-androgenic activity identified in environmental samples from the studies described above could be sufficient to induce effects in wild fish. Supporting this is one recent study of wild stickleback in the River Ray, which found that the concentrations of spiggin in females increased with the distance away from the WWTW effluent outfall. Moreover, when the treatment process at this WWTW was upgraded to a tertiary treatment, up to a two fold increase in spiggin in wild female stickleback was observed in comparison to measurements taken prior to the upgrade. Although this demonstrates anti-androgenic activity in wild fish, since spiggin production is androgen dependent, it does not necessarily constitute a negative impact on reproductive health (Katsiadaki *et al.*, 2012). Nonetheless, some adverse effects have been observed in exposures of fish to anti-androgenic effluent *in vivo*. For example, in stickleback exposed to an effluent with 328 µg/L FluEQ, reductions in male courtship behaviour and nest building were both observed. However, due to the lack of response in spiggin production it was considered that these effects may not have been due to anti-androgens and could have been impacted by the nature of the effluent. For example, its turbidity could have potentially affected visual signalling (Sebire *et al.*, 2011). Alternatively, behavioural effects could just have been a more sensitive endpoint than spiggin production, as observed during exposure to flutamide alone (Sebire *et al.*, 2008). In addition, two Australian studies which exposed fish to anti-androgenic WWTW effluent found potential impacts on the fecundity of male fish (Kumar *et al.*, 2012). In these studies, reductions or even absence of spermatozoa and increases in fibrotic tissue were found in the gonads of male zebrafish and Murray rainbowfish (*Melanotaenia fluviatilis*) exposed on site or

under laboratory conditions to effluents with strong anti-androgenic activity. Once again, in these examples, the cause of the activity remained unknown.

Crucially for this thesis, recent research has linked anti-androgenic activity to sexual disruption observed in wild roach in the UK (Jobling *et al.*, 2009). This used concentrations of steroid oestrogens, NP and its ethoxylates measured in UK effluents, as well as bioassay assessments of their (anti-) oestrogenic and (anti-) androgenic activity. It then modelled concentrations and activities occurring downstream of their outfalls at sites where fish were sampled as part of one of the nationwide surveys (Jobling *et al.*, 2006), and statistically modelled associations between the potential causes and effects. Unsurprisingly, this found that steroid oestrogens were strong predictors of vitellogenin induction, the occurrence of feminised ducts (ovarian cavities) and the incidence and severity of the oocytes in the testes. However, it also suggested that anti-androgens had a strong additive effect on these endpoints in combination with the oestrogens (see Figure 1.12 below for an example). In addition, anti-androgenic activity was also found to be significantly associated with vitellogenin induction alone. This has had implications for our knowledge of the causes of sexual disruption in wild fish, suggesting that the steroid oestrogens may be part of a bigger picture. So far there are no fully published *in vivo* studies to support this argument, although some preliminary data are available which indicates that a combination of anti-androgens detected in fish bile, including triclosan and chlorophene, could enhance the incidence of ovarian cavity induction in roach in combination with EE2. However, androgen induced spiggin production in female stickleback was not reduced by the mixture of these chemicals and so anti-androgenic activity was not confirmed in this species (Lange *et al.*, 2012a).

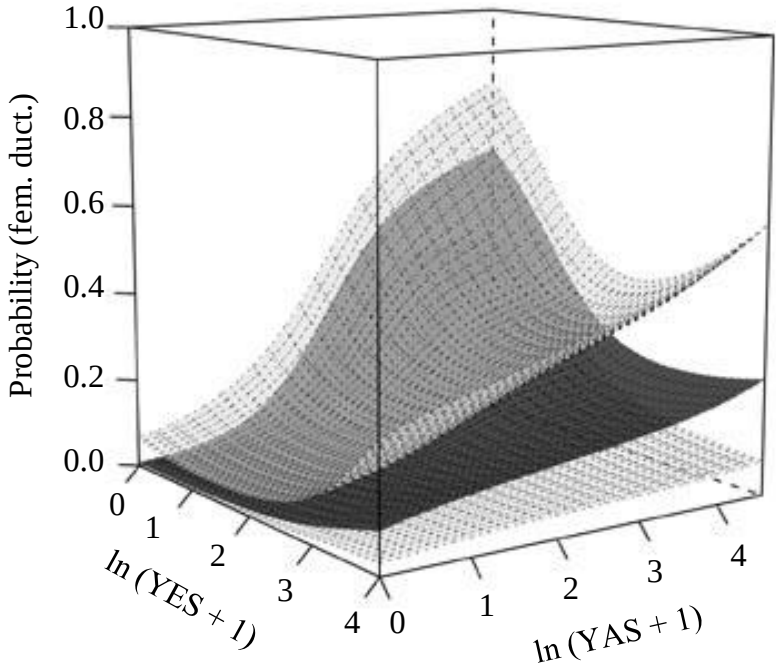


Figure 1.12 *A surface plot illustrating the results of statistical modelling of the association between exposure to oestrogenic (YES) and anti-androgenic chemicals (anti-YAS)* (from Jobling *et al.*, 2009)*. This indicates effects of anti-YAS alone and in combination with YES on the probability of feminised ducts in wild fish.*

1.8 Summary

EDCs are exogenous chemicals or mixtures which can negatively impact any aspect of the hormone system to cause adverse effects on an organism. They are ubiquitous pollutants which remain a cause for concern due to the increasing trends in endocrine related disorders in humans and observations of endocrine related effects in wildlife. In laboratory studies they have been linked to reproductive and developmental disorders, behaviour, obesity, cancers and immune disorders and new evidence is beginning to suggest that they can not only affect individuals, but may also have multigenerational and even population level impacts.

In fish, widespread oestrogenic activity in the environment has been strongly linked with sexual disruption observed in wild fish species globally. Steroid oestrogens are generally considered to be the major contributors, although some circumstances exist where other oestrogenic contaminants, such as alkylphenols, have also been linked to significant feminisation effects. However, anti-androgenic activity has recently been

found to be widespread in WWTW effluents in the UK and has also been detected in a number of other countries. In laboratory studies, anti-androgens can induce similar abnormalities to those caused by oestrogen exposure and those observed in the environment. Furthermore, it has been suggested that they may play a significant role in sexual disruption in wild fish alone or in combination with steroid oestrogens. Nonetheless, our understanding of environmental anti-androgens is still limited in comparison to the steroid oestrogens and it is important that they are identified and their impacts on fish are assessed, particularly in association with steroid oestrogens.

1.9 Thesis aim

The work presented in this thesis has contributed to a UK-Australian collaboration on EDCs in the aquatic environment. As a country, Australia has its own unique biota and climatic and environmental conditions which vary regionally. It is currently adapting to water scarcity and this may be exacerbated in the future by changing climate and its increasing population. Consequently, interest in EDCs in Australia has increased in recent years, in the context of climatic threats to the aquatic environment and its unique biota.

Wastewater treatment works effluent (WWTW) effluent contamination of the Australian aquatic environment is widespread across the country from temperate to tropical regions. Like in the UK, this can be characterised by a large contribution of effluent to river flow following discharge to small creeks or during periods of low rainfall, which can reach up to 100% downstream of WWTWs (Kumar *et al.*, 2012). In these effluents, oestrogenic activity is common and the steroid oestrogens have been detected at concentrations up to 54 ng/L E1 (Braga *et al.*, 2005), 18.5 ng/L E2 (Allinson *et al.*, 2010) and 1.3 ng/L EE2 (Ying *et al.*, 2009). Their concentrations show little variance between WWTWs in different regions with different climates, with similar concentrations detected in temperate South Australia, cool temperate Australian Capital Territory and subtropical Queensland (Williams *et al.*, 2007). They also compare well with the range of concentrations observed in the UK and other European countries (Johnson *et al.*, 2007b; Johnson *et al.*, 2005; Ying *et al.*, 2002b). In addition, other oestrogenic contaminants have also been detected, such as bisphenol A (BPA) and the alkylphenols, which are not subject to the same restrictions as in the EU (Ying *et al.*, 2009). Strong anti-androgenic activity was also detected in effluent from Queensland, which caused gonadal fibrosis and a reduction in spermatids in fish

exposures (Kumar *et al.*, 2012). However, there is little data available that quantifies this activity in environmental samples in Australia.

As a consequence of effluent discharge, oestrogenic activity has been detected in the aquatic environment and the concentrations of steroid oestrogens have been found to exceed their singular and combined PNECs for endocrine disruption in fish at WWTWs outfalls and downstream river sites (Ying *et al.*, 2009; Young *et al.*, 2004). However, concentrations have also been detected upstream of WWTW discharges, which suggests that multiple inputs should be considered. Indeed, in some localities such as small rural streams, agriculture has been shown to have a significant impact on steroid oestrogen concentrations (Williams *et al.*, 2007). How these discharges impact the aquatic environment is largely unknown, but there is already some evidence of estrogenic endocrine disruption in the pest species, eastern mosquitofish (*Gambusia holbrooki*), in effluent contaminated areas (Reitsema *et al.*, 2010; Rawson *et al.*, 2008; Batty and Lim, 1999). However, there have been no studies to determine the effects on wild native species, in part due to the limited understanding of their baseline biology, endocrinology and the lack of available tools for ecotoxicology testing. This situation is improving and recent research has developed tools for measuring vitellogenin in the barramundi (*Lates calcarifer*) and black bream (*Acanthopagrus butcheri*) (Codi King *et al.*, 2008), which found that they were susceptible to oestrogen exposure. Work has also been completed on the Murray rainbowfish (*Melanotaenia fluviatilis*) to assess baseline reproduction and the use of molecular methods and biomarkers has also been investigated (Woods and Kumar, 2011; Pollino *et al.*, 2007; Pollino and Holdway, 2003). This found that these fish could be affected by oestrogens and consequently, the Murray rainbowfish has been proposed for use as an Australian fish model to assess endocrine disruptors. Nonetheless, so far there have been no assessments of intersex in wild native fish in oestrogen contaminated areas. Indeed, the closest example is a study of invasive common carp on the River Yarra, which found no evidence of vitellogenin induction or gonadal abnormalities. However, this was unsurprising due to the dilution of tertiary treated effluent within this catchment, which resulted in no oestrogenic activity being detected at capture sites (Kumar *et al.*, 2012).

In both the UK and Australia, anti-androgenic activity occurring in the environment is an emerging topic. The causes remain largely unknown and so it is important that significant environmental anti-androgens are identified in both countries and their effects assessed in fish models. Additional consideration should also be made for the possibility of mixture effects with steroid oestrogens on common endpoints. Consequently, the overall aim of this thesis project was to identify anti-androgens in UK

and South Australian catchments and to produce environmentally relevant exposures to assess their impacts on sexual disruption in fish alone and in combination with steroid oestrogens. The hypothesis was that anti-androgens would be detected in the environment and that they could cause sexual disruption in fish under experimental conditions. The null hypothesis was that anti-androgenic compounds would not be detected in the environment and that antiandrogens would not affect sexual disruption in fish *in vivo*. Work towards the overall project aim was completed under three objectives:

Objective One: Predictive modelling of anti-androgenic pharmaceuticals

This represented a targeted approach to identifying environmental anti-androgens by focussing on a set of pharmaceuticals with an anti-androgenic mode of action. Effluent and river concentrations of these pharmaceuticals were estimated in a UK and a South Australian catchment using predictive modelling techniques. Concentrations of steroid oestrogens were modelled alongside the anti-androgenic pharmaceuticals. The models were then adapted to analyse future trends in their concentrations and associated risks to fish health under climate and population change scenarios up to 2050.

Objective Two: *In vivo* assessment of pharmaceuticals

The two major anti-androgenic pharmaceuticals, bicalutamide and cyproterone actetate, were applied in two *in vivo* assessments with two appropriate fish models. The exposure concentrations were informed by the predictive modelling in objective one to produce environmentally relevant exposure scenarios. This aimed to determine the impacts of these pharmaceuticals on sexual disruption alone and in combination with steroid oestrogens, using endpoints including vitellogenin induction, secondary sexual characteristics and intersex.

Objective Three: Effect Directed Analysis of WWTW effluents

As part of a broader approach to identifying anti-androgens, the chemical constituents of UK WWTW effluent and river samples were identified following extraction, fractionation and gas chromatography-mass spectrometry. Potential anti-androgens were identified through literature searches and *in vitro* bioassays and their contribution to the identified activity was determined.

CHAPTER 2: PREDICTING CONCENTRATIONS OF ANTI-ANDROGENIC PHARMACEUTICALS AND STEROID OESTROGENS IN WWTW EFFLUENTS AND RIVER CATCHMENTS

2.1 Introduction

Pharmaceutically active chemicals have become contaminants of emerging concern in the last decade as a result of their widespread presence in the environment and their potential ability to interact with non-target organisms. Endocrine disruption in wild fish is a major example of this, having been linked to the steroid oestrogens 17α-ethinylestradiol (EE2), 17β-oestradiol (E2) and oestrone (E1). As natural ligands for the oestrogen receptor, E1 and E2 are highly active chemicals in comparison to xenoestrogen mimics both *in vitro* and *in vivo* (Metcalfe *et al.*, 2001; Harris *et al.*, 1997; White *et al.*, 1994). However, the pharmaceutical EE2 remains the most potent steroid oestrogen *in vivo* as a designed oestrogen receptor agonist with the addition of an ethinyl group which reduces its rate of metabolism (Routledge *et al.*, 1998). This is reflected in the most recently proposed predicted no effect concentrations (PNECs) of 0.1, 2 and 6 ng/L for EE2, E2 and E1 respectively, derived from reproductive endpoint data from fish studies and supplemented with data on vitellogenin induction (Caldwell *et al.*, 2012).

Based on these data, it is plausible that some of the most active environmental endocrine disruptors with anti-androgenic activity could be pharmaceuticals with an androgen receptor (AR) antagonistic mode of action. Consequently, investigating the presence of anti-androgenic pharmaceuticals in the environment presented the possibility for a targeted approach to identifying and assessing environmental anti-androgens. One set of methods that can be used to make these assessments are predictive modelling techniques, which have been successfully applied to the issue of environmental steroid oestrogens and their risk assessment in the UK (Williams *et al.*, 2009). These methods provide fast, preliminary risk assessment tools and therefore provide a good starting point for assessing these pharmaceuticals and their potential risk to wild fish in river catchments. However, in Australia, predictive modelling and its associated risk assessments have not yet been utilised since the hydrological models to enable this process have not been developed. Nonetheless, their development could prove to be highly informative in assessing concentrations of anti-androgens and steroid oestrogens when applied to catchments in this water stressed continent. Indeed, the development of desktop based modelling of endocrine disrupting chemical (EDC) exposure in Australia is one of the research priorities identified in the Black Mountain Declaration. This originated from a workshop on EDCs incorporating researchers, policy makers, regulators, water suppliers and research investors. Moreover, the anticipated global population growth during this century alone (United Nations, 2011), coupled with climate induced changes in precipitation (Bates *et al.*,

2008), provides an additional need to assess the consequences of changing water availability on EDC concentrations to ensure that any mitigation options proposed are of an appropriate scale to be effective in the longer term.

2.1.1 Pharmaceuticals as emerging contaminants

Pharmaceuticals are complex molecules with a relatively specific biological activity, which enter the aquatic environment following patient excretion and incomplete removal during wastewater treatment. In addition, hospitals, manufacturers, landfills and improper disposal by patients can also be prominent sources (Kümmerer, 2009; Larsson *et al.*, 2007; Bound and Voulvoulis, 2005). Due to their constant use they are also constantly released into the aquatic environment. Their concentrations have a tendency to be positively correlated to prescription frequency and are generally within the ng/L to µg/L range. Whilst they can occur in their parent form, some may form active metabolites and biotransformation products through human or bacterial metabolism or environmental degradation. Due to their widespread and increasing use globally, pharmaceuticals have been detected in WWTW effluent, surface waters, ground water and drinking water (Heberer, 2002). Indeed, a nationwide US survey found that the occurrence of pharmaceuticals in sampled streams was widespread (Kolpin *et al.*, 2002a). This study achieved significant interest from both the public and the scientific community, highlighting the issue of pharmaceuticals as emerging contaminants of concern. However, it also caused some controversy due to the high concentrations of EE2 reported, with a maximum concentration of 831 ng/L (later modified to 273 ng/L) (Kolpin *et al.*, 2002b). This greatly exceeded values reported elsewhere in the literature, which in review ranged from <0.5 – 15 ng/L and it was suggested that this could be caused by another substance interfering during chemical analysis (Ericson *et al.*, 2002). Nonetheless, the concentrations of other drugs, such as ibuprofen and fluoxetine, compared well with a review of pharmaceuticals in the environment, which demonstrates the range of drugs that have been identified (Corcoran *et al.*, 2010).

There is concern that some drugs could induce adverse effects in non-target organisms through their specific interactions with conserved drug targets (Rand-Weaver *et al.*, 2013; Gunnarsson *et al.*, 2008). Indeed, drug targets such as steroid receptors and the hypothalamic-pituitary-gonadal (HPG) axis are highly conserved between vertebrates, increasing the plausibility of pharmaceutical interactions in other species (LaLone *et al.*, 2013; Perkins *et al.*, 2013; Gunnarsson *et al.*, 2008). As a result, groups of

pharmaceuticals that interact with steroid receptors other than the oestrogen receptor, including anti-androgens, are also in significant use by the human population and have been recommended as high priorities for research (Runnalls *et al.*, 2010). In many cases research suggests that pharmaceutical concentrations in the aquatic environment tend to be at least one magnitude lower than the concentrations required *in vivo* (µg/L to mg/L) to cause effects in fish. However, there are some exceptions, such as EE2 and ibuprofen (Corcoran *et al.*, 2010). Indeed, EE2 is being considered for regulation under the European Water Framework Directive with its addition to the list of "priority substances" by the European Commission. In contrast, there is no data available on the concentrations of anti-androgenic pharmaceuticals in UK or Australian WWTW effluents and surface waters. These pharmaceuticals are prescribed in both countries to treat androgen dependent disorders and they have been suggested as high priorities for research due to their prescriptions and mode of action (Runnalls *et al.*, 2010). They can be split into two classes: pure, non-steroidal anti-androgens and steroidal derivatives (Singh *et al.*, 2000).

2.1.1.1 Non-steroidal anti-androgens

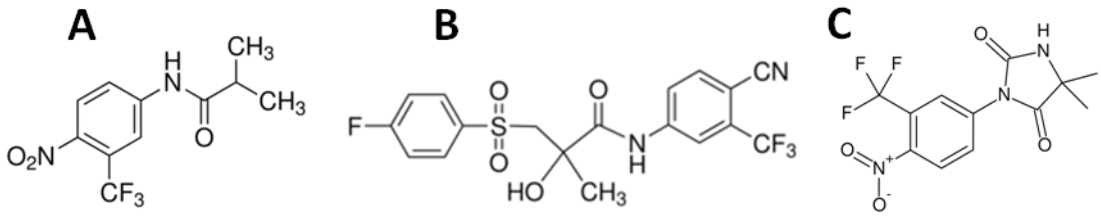


Figure 2.1 *The non-steroidal anti-androgens: flutamide (A), bicalutamide (B) and nilutamide (C)* (sourced from Sigma-Aldrich).

The non-steroidal anti-androgens (Figure 2.1) are pure AR antagonists. They do not exert any agonistic activity on the receptor, nor do the show any binding capability with other nuclear receptors (Singh *et al.*, 2000). In human medicine, they are all used to treat androgen dependent advanced prostate cancer, the second most common cause of cancer deaths in men. They are generally able to preserve sexual function but gynecomastia can be a problematic side effect (Mahler *et al.*, 1998). Out of the three, nilutamide is not prescribed in the UK and flutamide has been recently discontinued by the NHS in favour of the prescription of bicalutamide. Nonetheless, all three pharmaceuticals are still prescribed in Australia.

Both flutamide and bicalutamide have been subject to *in vivo* testing with fish models, but there is no data available for nilutamide. The majority of *in vivo* data exists for flutamide, which has been used as a model anti-androgen in a number of studies, where it has been shown to cause reductions in secondary sexual characteristics and fecundity (Jensen *et al.*, 2004; Panter *et al.*, 2004), changes to reproductive behaviour (Sebire *et al.*, 2008) and intersex (León *et al.*, 2007; Kang *et al.*, 2006). In contrast, there is only one published exposure study of bicalutamide, which also caused reductions in secondary sexual characteristics and reproductive parameters (Panter *et al.*, 2012). Both pharmaceuticals induced these effects in the µg/L range. Whilst flutamide produces an active metabolite, hydroxyflutamide, bicalutamide is a racemic mixture with anti-androgenic activigy occurring almost exclusively in the R enantiomer (Cockshott, 2004).

2.1.1.2 Steroidal anti-androgens

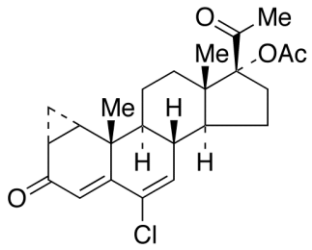


Figure 2.2 *The steroidal anti-androgen, cyproterone acetate* (sourced from TRC Canada).

As well as binding the AR antagonistically, cyproterone acetate (Figure 2.2) also has some agonistic activity and interacts with other hormone receptors. In fact, it can also block gonadotropin secretion through its gestagenic activity and can therefore reduce the availability of circulating androgens, as well as blocking androgen activity at the target site. Consequently, cyproterone acetate has multiple uses, as well as the treatment of prostate cancer. These include the treatment of hirsuitism and acne in women, where is it prescribed in conjunction with EE2 in a combined contraceptive pill, and in the treatment of sexual deviation in males (Unluhizarci *et al.*, 2013; Arowojolu *et al.*, 2012; Schneider, 2003; Bradford, 1999; Mahler *et al.*, 1998). Like flutamide, it also has an active metabolite: 15β-hydroxy-cyproterone (Huber *et al.*, 1988). Cyproterone acetate is prescribed in both the UK and Australia. *In vivo* exposures of fish models have been completed and like the non-steroidal anti-androgens, cyproterone acetate

also caused intersex and other gonadal abnormalities, as well as changes to plasma sex steroid and urinary metabolite profiles and male parental behaviour (Dey *et al.*, 2010; Collette *et al.*, 2010; Kiparissis *et al.*, 2003) in the µg/L range. However, there is also some evidence of effects on plasma sex steroid concentrations in both males and females in the ng/L range (Sharpe *et al.*, 2004), which may be closer to environmental concentrations.

2.1.2 Predictive modelling of contaminants in the environment

Identifying at risk regions where pharmaceuticals pose a risk to environmental and human health at present or in the future is one of the top 20 research questions regarding these contaminants (Boxall *et al.*, 2012). Predictive modelling of river catchments is one of the methods which can provide the tools to do this, potentially at a national scale, detecting "hot spots" of chemical contamination, which could pose a threat to aquatic biota. In European rivers, the concentrations of steroid oestrogens (Williams *et al.*, 1999), pharmaceuticals (Kugathas *et al.*, 2012; Schowanek and Webb, 2002) and chemicals originating from personal care products and household cleaners (Wind *et al.*, 2004) have all been assessed by predictive modelling. These techniques have been used alongside or as an alternative to analytical chemistry and have the advantage of being cheap and fast first tier assessment tools. In comparison, analytical chemistry is an expensive and time consuming process and detection limits can provide difficulties, particularly with substances which are highly active at low concentrations. This has been a particular issue with EE2, where a limit of detection below the environmental quality standard of 0.1 ng/L is required for robust analysis. In many cases this can only be achieved through extraction of large sample volumes, which becomes even more time consuming (Johnson *et al.*, 2008). However, producing more complex river catchment models is also a time consuming process and requires extensive data and specialists to program them in their initial set up. The output of these models is also only as good as their input data, so time investment at this point is crucial. Once a catchment model has been set up it can be easily used to assess multiple chemicals simultaneously and they can be modified to assess different environmental scenarios, such as the effects of perturbations like climate change (Johnson *et al.*, 2008). Nonetheless, it should be considered that whilst modelling procedures work well as a guide, it is only robust analytical chemistry that can confirm the presence of a chemical in the environment (Johnson *et al.*, 2008).

In a basic modelling procedure, data are required on the consumption of a chemical and the population served at a WWTW to determine the chemical load arriving at the works. The effluent flow and potential removal rates are then taken into account to determine the effluent concentration and the receiving river flow can be used to calculate concentrations in surface water downstream of the outfall. Some of these techniques were pioneered in the UK to assess concentrations of steroid oestrogens. Here, Johnson and Williams produced a method to determine the amount of each steroid oestrogen entering a WWTW based on their excretion from different cohorts within the human population (Johnson and Williams, 2004; Johnson *et al.*, 2000). This was used as the basis for predictions of effluent concentrations as well as river concentrations at capture sites from the UK survey of intersex roach, which were based on the available effluent dilution (Jobling *et al.*, 2006). This study identified significant relationships between the predicted concentrations of steroid oestrogens and both the prevalence and severity of intersex within the captured roach, adding further evidence to the hypothesis that steroid oestrogens were significant factors in the causation of sexual disruption. Following on from this, a national risk assessment of steroid oestrogens was completed using the Johnson and Williams model in conjunction with a geographic referenced point source water quality model: the LowFlows 2000 Water Quality eXtention Model (LF200-WQX). This is a digitised river network which incorporates WWTW discharge points as well as confluences, bifurcations, abstraction points and runoff data to simulate the input and dilution of contaminants (Williams *et al.*, 2009). In this study, the model included 10,313 river reaches (21,452 km length) in England and Wales with 2,000 WWTWs which served over 29 million people. It produced a map of oestrogen concentrations on these stretches and found that 39% were potentially at risk of endocrine disruptive effects by exceeding the 1 ng/L combined E2 equivalent on average (Young *et al.,* 2004). It also used data from fish exposure studies and PNECs to determine a risk class for each stretch based on the E2 equivalent concentration of the three steroid oestrogens, which is demonstrated below in Figure 2.3. In addition, further investigation of this hydrological modelling technique on the Rivers Erewash and Avon found that measurements by analytical chemistry of samples taken from along the river adequately matched the predicted concentrations in terms of their risk level (Williams *et al.*, 2012).

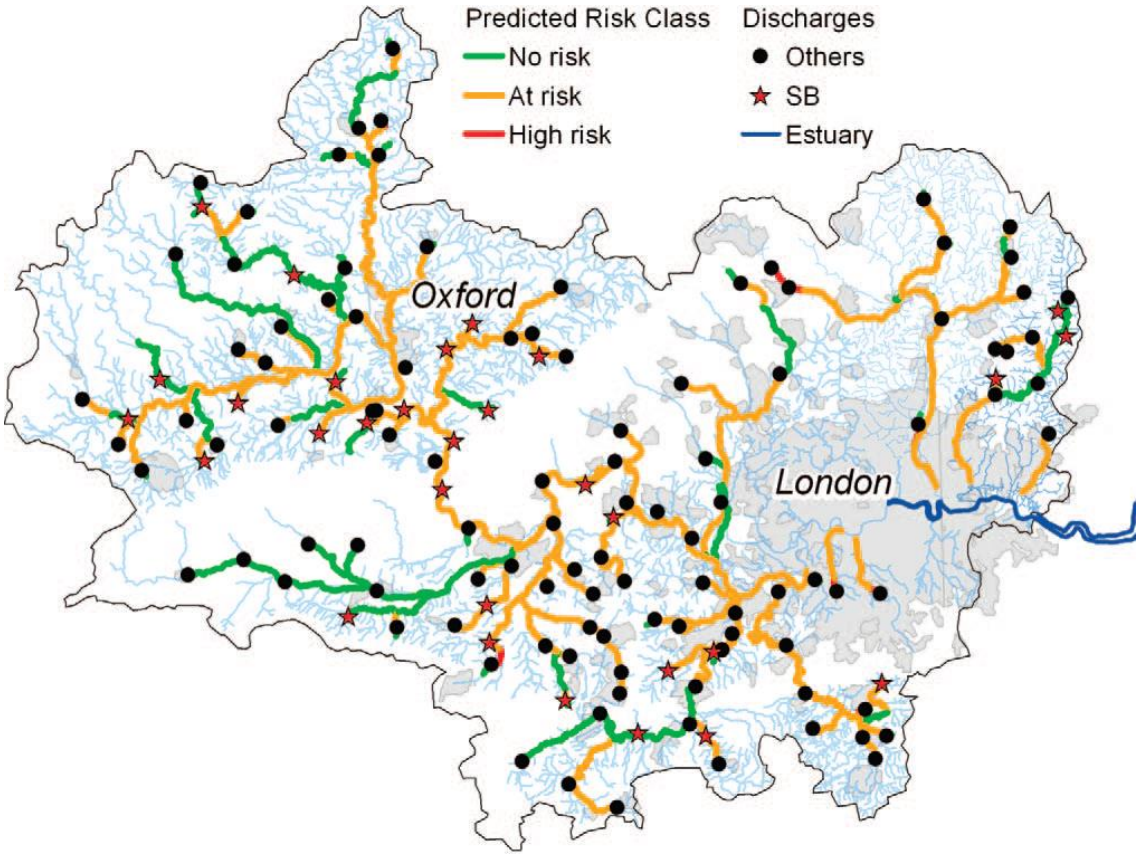


Figure 2.3 *The predicted risk classes of modelled river stretches in the Thames region based on the E2 equivalent concentration of the steroid oestrogens combined. No risk (<1 ng/L), at risk (1-10 ng/L) and high risk (>10 ng/L). SB denotes WWTWs using a secondary biological filter as a treatment process* (from Williams *et al.*, 2009)**.**

These techniques have also been applied to investigate a range of mitigation options at WWTWs in the UK (Johnson *et al.*, 2007a), as well as the mixture effects of oestrogens and xenoestrogens in a UK river catchment (Sumpter *et al.*, 2006). Similar modelling studies have also been completed in Japan and the USA where lower concentrations and therefore risk of endocrine disrupting effects occurring in fish were predicted. This is likely to be due to the significantly greater dilution available per capita in both countries, although in Japan, the lower use of the contraceptive pill was also a smaller contributing factor. Indeed, in the USA only effluent dominated streams with low dilution were considered to be at risk of endocrine disruption (Anderson *et al.*, 2012; Johnson *et al.*, 2011).

2.1.3 Predicting future changes in environmental EDC concentrations

Human population density and the available dilution in the aquatic environment are key factors responsible for producing concentrations of environmental contaminants (Williams *et al.*, 2009; Jobling *et al.*, 2006), both of which are predicted to dramatically change during the course of this century. Indeed, many countries are anticipating increasing population pressure with the global population expected to reach 9.3 billion by 2050. This will put increasing pressure on WWTWs as the population they are required to serve increases, which will in turn increase the anthropogenic pollutant load that will require treatment before release into the aquatic environment. This is likely to occur in the context of increasing demand on water resources, which for many catchments are already considered to be under stress. In fact, nearly 80% of the global population is estimated to be exposed to a high threat to their water security (Vorosmarty *et al.*, 2010), which could increase reliance on treated wastewater for domestic use, potentially leading to greater human exposure to contaminants (Kookana *et al.*, 2007). In both the UK and Australia, many regions are already experiencing degrees of water stress and management plans are being enacted to ensure that water is adequately supplied to both the human population and the environment (National Water Commission, 2012; Government of South Australia, 2010; Environment Agency, 2008c; HM Government and DEFRA, 2008).

In the future, in some regions the anticipated effects of climate change include reductions in precipitation, runoff and river flows, adding to water stress and reducing the available dilution of the increasing contaminant load (Bates *et al.*, 2008). Indeed, widespread reductions in total annual river flow of 10-15% are expected throughout England and Wales by the 2050's based on low to high emissions scenarios (Environment Agency, 2008a). This compares well with a more specific study focussing on the effects of low to high emissions scenarios on the River Thames and the Ouse by 2080, which anticipated flow reductions in all seasons, except winter on the Ouse, and a decrease in the duration of high flow events. However, on the Thames the reduction in flow was predicted to be higher than those projected in the national report (30-50%) (Johnson *et al.*, 2009). In Australia, flow on the Murray River, Australia's largest river basin which accounts for 70% of irrigated crops and pastures, is predicted to decrease by 10-25% by 2050 and 16-48% by 2100 (Bates *et al.*, 2008; Beare and Heaney, 2002). Reductions have also been predicted in south west and eastern Australia, although an increase in runoff is expected to occur in northern Australia (Milly *et al.*, 2005). As well as a reduction in baseline flows, reducing precipitation and runoff is also anticipated to increase the frequency of drought events. Although both countries

already experience drought, increasing frequency, intensity and their spatial extent will impact environmental pollutants and add a significant challenge to water resource management in the future (Rahiz and New, 2013; Fowler *et al.*, 2003; Hughes, 2003).

To maintain river flows that will be sufficient to sustain environmental health and suit anthropogenic need, the importance of WWTW effluent flow will increase, as will potential trade-offs between water quality and quantity (Land and Water Australia, 2008). This will have implications for aquatic biota, for which biodiversity loss is linked to reduced flows (Xenopoulos *et al.*, 2005). In the UK, one study found that low dissolved oxygen and increased water temperature associated with low flows were linked to migratory decline in Atlantic salmon. In this case over 50% of the salmon run was lost during these low flow events (Solomon and Sambrook, 2004). Nonetheless, Australian data suggests that there may be some advantages to low flow scenarios for recruitment of some species due to the concentration of prey species, such as zooplankton, during the larval stage (Humphries *et al.*, 1999). However, early development is very sensitive to endocrine disruption by pollutants and for many aquatic species this life stage occurs during periods of low flow in summer months, when contaminant concentrations are likely to be at their highest. Consequently, to ensure that appropriate management action and mitigation responses are made, it is important that endocrine disruptors and their risks to fish in the future are properly assessed under multiple scenarios.

2.1.4 Aims and objectives

This study aimed to investigate the input of anti-androgenic pharmaceuticals into the aquatic environment via WWTW effluents and their resultant concentrations in a river catchment in the UK and one in South Australia using predictive modelling techniques. This represented a targeted approach to identifying environmental anti-androgens, in which the concentrations were compared with *in vivo* data to determine their potential risks to wild fish health. Since these techniques have not yet been employed to assess EDCs in Australia, concentrations of steroid oestrogens were modelled alongside the anti-androgenic pharmaceuticals. This would also allow combined exposures of fish to anti-androgens and steroid oestrogens, detailed in Chapter 3, to be informed by appropriate concentrations. Finally, the projected changes in climate and human populations in the future provided the impetus to assess how contamination of river catchments by these chemicals could change in both countries up to 2050. The

impacts of contamination of these catchments on fish in both the present day and future scenarios were also explored.

2.2 Materials and methods

2.2.1 Sites

Four UK WWTWs (UK1-4) located in the Severn-Trent catchment was compared with 12 WWTWs in South Australia (Table S1). Both represented a variety of rural and urban scenarios and both catchments are considered to be moderately water stressed, since the demand and allocation of water is a high proportion of the total availability (National Water Commission, 2012; Government of South Australia, 2010; Environment Agency, 2008c; HM Government and DEFRA, 2008). The river hydrology of the two catchments contrast with cooler, permanently flowing waters in the UK and warmer more ephemeral hydrology dominated by winter flow in South Australia.

2.2.2 Modelling pharmaceuticals

The annual consumption of anti-androgenic pharmaceuticals and EE2 were calculated based on the number of prescriptions in the UK and Australia, which were determined from the National Health Service's Prescriptions Cost Analysis (2009) for England (The NHS Information Centre, Prescribing Support Unit, 2010) and Wales (Welsh Assembly Government, 2010) and the Australian Statistics on Medicines (2008) (Australian Government Department for Health and Aging, 2009), using a method from Runnalls *et al.* (Runnalls *et al.*, 2010). The per capita load in µg/d was then determined based on the annual consumption, the population of each country for the prescription year and the excretion rate of the chemical. These are shown in Table 2.1 below. This assumes that patient compliance with their presciptions is 100%, in that all of the prescribed pharmaceuticals are consumed. Nonetheless, given that these drugs are prescribed as cancer treatment or contraception it could be considerd that compliance is likely to be high.

Table 2.1 *The prescriptions of pharmaceuticals in the England and Wales (2009) and Australia (2008).*

Pharmaceutical	Consumption (kg) England and Wales (2009)	Consumption (kg) Australia (2008)	Excretion %[1]	Per capita load (µg/day)[2] England and Wales (2009)	Per capita load (µg/day)[2] Australia (2008)
Bicalutamide	635.77	53.91	55.00%	17.43	3.68
Cyproterone acetate	278.90	361.21	80.75%	11.23	36.22
Flutamide	146.52	70.83	39.90%	2.91	3.51
Nilutamide	-	7.05	100%	-	0.86
17α-ethinylestradiol	17.42	5.55	40.00%	0.35	0.28

[1] *Excretion rates based on average urinary and biliary excretion of bicalutamide* (Goa and Spencer, 1998)*, cyproterone acetate* (Frith and Phillipou, 1985; Humpel *et al.*, 1977; Speck *et al.*, 1976)*, flutamide* (Wolters Kluwer Health, 2009; Schering-Plough Pty Limited, 2004; Solimando, 1997) *and EE2* (Johnson and Williams, 2004)*. With no data available for nilutamide, a worst case scenario of 100% excretion was assumed.*

[2] *Based on a mid-2009 population of 54,809,100 for England and Wales and a 2008 population of 22,000,000 for Australia from the ONS and ABS.*

2.2.3 Modelling natural oestrogens: oestrone (E1) and 17β-oestradiol (E2)

This model was based on an approach provided by Johnson and Williams, which has been applied to predict environmental concentrations of steroid oestrogens in effluents in Europe (Johnson and Williams, 2004), as well as in hydrological models used for national risk assessments of endocrine disruptors in rivers in the UK, Japan and the USA (Anderson *et al.*, 2012; Johnson *et al.*, 2011; Williams *et al.*, 2009). Our modified model provides a per capita load for E1 and E2 in µg/d arriving at a WWTW, based on the proportions of different oestrogen-excreting cohorts within a population. This was calculated as follows:

$$SE2 = 0.5 \sum_{i=1}^{n} fi\ (UE2)$$

$$SE1 = \sum_{i=1}^{n} fi\ (UE1) + 0.5\ SE2$$

Where *S* is the per capita load arriving at a WWTW (µg/d), *n* is the number of cohorts and U is the total oestrogen excreted in urine (in free, glucuronide and sulfate forms) and faeces for each cohort percentage (*fi*) of the population (Table 2.2). For E2, a factor of 0.5 is incorporated assuming that 50% will be degraded to E1 in transit through the sewerage system to a WWTW. The mean oestrogen excretion of each cohort percentage is shown in Table 2.2 and is based on a literature review for the original model that focused on Caucasian omnivorous women (Johnson and Williams, 2004). Upper and lower excretion values were also used to provide a range in the load arriving at a WWTW. A worked example can be found in the Appendix One.

Table 2.2 *The population breakdown of the oestrogen excreting cohorts by criteria and the composition of each census population: UK 2001 and Australia 2006.*

Cohort	Criteria	Mean (range) excretion (µg/d)		% of population	
		E2	E1	UK	Australia
Menstrual females	Age 15-50 (minus pregnant women)	3.2 (1.7-4.6)	11.7 (7.5-15.4)	23.5%	24.2%
Menopausal females	Age >51 (minus menopausal women on HRT)	1 (0-3.5)	1.8 (0-5.7)	16.1%	13.7%
Menopausal females on HRT	7.6% UK and 11.8% Australian menopausal females (>51)	56.1 (51.5-61.5)	28.4 (24-33)	1.3%	1.8%
Pregnant Females	1/22 UK and 1/19 Australian menstrual females	393 (340-445)	550 (432-668)	1.1%	1.3%
Males	Age >15	1.8 (1.3-2.4)	2.6 (1.4-2.9)	39.0%	39.2%

2.2.3.1 Cohort Criteria

The percentages of the populations made up by each cohort were based on age and determined from national census data, which were assumed to be relevant to local demographics. This utilised the national report for England and Wales (age by sex and resident type) from the 2001 census by the Office for National Statistics (ONS) and the Australian 2006 census (age by sex based on place of usual residence) from the

Australian Bureau of Statistics (ABS). Pre-pubescent males and females were not incorporated since sex steroid production is low until puberty and their inclusion would have little effect on the final prediction (Johnson and Williams, 2004). As a result, the male cohort included those from age 15 onwards and menstrual females were assumed to be between 15 and 50 with menopausal females taken from the age of 51 onwards. The number of females on hormone replacement therapy (HRT) using E2 based pharmaceuticals was updated for this model where 11.8% of women over 50 were estimated to use HRT in Australia (MacLennan *et al.*, 2009) compared to 7.6% of women in the UK. This was calculated by combining population data from the 2001 census with data on HRT use in the UK in 2004 (Watson *et al.*, 2007). These percentages were applied to the menopausal female cohort to determine the number of women on HRT, although it should be taken into account that HRT use has fluctuated in the last decade in both countries (MacLennan *et al.*, 2009; Watson *et al.*, 2007). The number of pregnant females was estimated using the census data assuming that the number of live births (people aged 0) was representative of the number of pregnant females. Using this model, per capita loads of 3.4 (2.7-4.1) and 3.9 (3.2-4.7) µg/d were produced for E2 in the UK and Australia respectively, as well as 14 (10-18) and 16 (12-20) µg/d for E1.

2.2.4 Predicting concentrations in WWTW effluent

The linear emission model was used to predict effluent concentrations (µg/L) of the pharmaceuticals and steroid oestrogens, which reflected a 24-hour composite sample of effluent. WWTW data used in this model is shown in Table 2.3 below. The total load arriving at a WWTW (the per capita load [*pc*] (µg/d) multiplied by the population *(pop)* serviced) was divided by the total flow *(Q)* (L/day) through the WWTW (domestic plus non-domestic flow). Since removal is reported to be low for bicalutamide (Brixham Environmental Laboratory, 1998) and no data was available for the other anti-androgenic pharmaceuticals, a removal percentage *(R)* was not incorporated. This provided a worst case scenario where the total pharmaceutical load arriving at a WWTW was available to enter the aquatic environment. In contrast, removal rates of 69% and 83% were incorporated for E1 and E2 respectively, based on a review of removal during the activated sludge process (ASP) (Williams *et al.*, 2008). However, it should be recognized that in reality removal rates vary, even in a single WWTW, based on the treatment process and environmental conditions (Johnson *et al.*, 2008). Flow and population data for the WWTWs were provided by Severn Trent Water, UK and South Australian Water Corporation, Australia (Table 2.3).

$$C_{effluent} = \frac{pc \cdot pop}{Q_{STW}} \cdot (1 - R)$$

Average, upper and lower effluent concentrations for E1 and E2 were produced by varying the per capita loads with the upper and lower excretion values (Table 2.2), whilst for EE2 different removal rates during the activated sludge process (83%, 71.2% and 94.8%) were assumed.

Table 2.3 *The parameters for UK and South Australian WWTW for the present day***.**

WWTW	Population Served	Domestic Flow (Mega Litres/Day)	Non-Domestic Flow (Mega Litres/Day)	Total Flow (Mega Litres/Day)	Per capita flow (L/Day)
UK1	53,695	8.17		8.17	152
UK2	47,991	13.52		13.52	282
UK3	117,041	38.60		38.60	330
UK4	68,300	11.41		11.41	167
SA1	2,761	0.23	0.05	0.28	102
SA2	4,163	0.86		0.86	206
SA3	9,294	1.79		1.79	193
SA4	3,300	0.76		0.76	230
SA5	9,965,58	145.03	11.35	156.38	157
SA6	17,652	5.24		5.24	297
SA7	1,029	0.26		0.26	257
SA8	2,873	2.45		2.45	852
SA9	226	0.06		0.06	273
SA10	2,725	0.36		0.36	134
SA11	3,571	1.12		1.12	313
SA12	16,159	4.36		4.36	270

[1] The population serviced at UK WWTW's was based on estimations from Severn Trent Water for the year 2010. At Australian WWTW's, the average population was determined based on estimations from the years 2005-10.

[2] Flow rates at UK WWTW's represent the average daily flow for the year 2010 provided by Severn Trent Water. Australian WWTW's used the average flow rate from the years 2005-10.

2.2.5 The relevance to real world effluents

Since there is little data available on the concentrations of the anti-androgenic pharmaceuticals in effluents, comparisons were made between modelled and measured steroid oestrogens to determine the relevance of modelled data to real world concentrations in effluent. Modelled concentrations were compared with measured data from UK2, where data from 19, 24-hour composite samples of its activated sludge treated effluent were available from a previous study (Baynes *et al.*, 2012). These were collected between July and December 2009 and analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (Kanda and Churchley, 2008). These data were compared with daily average modelled concentrations based on flow data from UK2 provided by Severn Trent Water from the day of sampling (Table 2.4). Up to 96 flow measurements were taken daily so concentrations were produced for each flow rate based on the average per capita loads.

Table 2.4 *Average (minimum – maximum) flow rates from UK2 at sampling points between July and December 2009, provided by Severn Trent Water. No data was available from 27/8/09 to 21/9/09.*

Sampling Date	Flow (Mega litres/day)	Sampling Date	Flow (Mega litres/day)
10/07/2009	205 (95-620)	01/10/2009	190 (70-419)
16/07/2009	362 (197-590)	06/10/2009	323 (77-638)
22/07/2009	350 (213-591)	13/10/2009	173 (75-285)
27/07/2009	331 (153-630)	23/10/2009	202 (82-369)
05/08/2009	489 (334-629)	26/10/2009	185 (87-291)
14/08/2009	230 (110-367)	04/11/2009	389 (269-618)
18/08/2009	212 (95-361)	19/11/2009	407 (362-577)
27/08/2009	-	20/11/2009	339 (191-602)
02/09/2009	-	24/11/2009	465 (359-605)
10/09/2009	-	02/12/2009	452 (366-603)
18/09/2009	-	07/12/2009	506 (439-570)
21/09/2009	-	15/12/2009	403 (290-629)

2.2.6 Predicting river concentrations

2.2.6.1 UK, Low Flows 2000-WQX

The LF2000-WQX model (Wallingford Hydrosolutions, UK) was used to predict concentrations of anti-androgenic pharmaceuticals and steroid oestrogens in the River Erewash (as described by Williams *et al.*, 2009). LF2000-WQX provided a map of interconnected river reaches, a digitised river network, with artificial influences (e.g. abstractions and discharges) incorporated. Since gauging sites were not available to provide flow data for all modelled river stretches, the magnitude and variability of flows at ungauged sites were estimated from runoff and generalized against gauged sites. This was achieved using a statistical probability approach where Monte-Carlo simulations generated a flow distribution based on different flow rate scenarios from extensive historical flow and rainfall data (Johnson *et al.*, 2008). The concentrations predicted by this modelling procedure were therefore output as a distribution. The mean was used in this study since it represented the predicted concentrations for average river conditions.

The chemicals were assumed to enter the system continuously via the eight WWTWs on the Erewash including UK2 and 4. The per capita load arriving at these WWTWs was based on the effluent model with serviced populations updated with new estimates from Severn Trent Water. The dry weather flows (DWF) through the WWTWs in the LF2000-WQX model were updated in line with the population to maintain the per capita flow. Again, no removal was assumed for the anti-androgenic pharmaceuticals, whilst removal of steroid oestrogens at each WWTW was based on the ASP review used in the effluent model (Williams *et al.*, 2008). The average concentrations on a given stretch were then determined based on an exponential decay model incorporating in-stream temperature dependent degradation for steroid oestrogens only (Jürgens *et al.*, 2002) and dilution based on the spatial variability in flow. Loss through absorption to sediment was not included since it is not a cause of significant removal for oestrogens (Holthaus *et al.*, 2002). Degradation of E2 to E1 was also incorporated based on 1 mol E2 degrading to 1 mol of E1. However, in the case of the anti-androgenic pharmaceuticals, the model produced a worst case scenario for their environmental concentrations based on average river flow, since no data was available on the in-stream degradation of the drugs. Consequently, only the effect of dilution of the effluent in downstream river stretches was taken into account.

2.2.6.2 South Australia, Source Catchments

A point source hydrological model of the Onkaparinga River in South Australia was implemented and run in Source Catchments version 2.0.4 (eWater CRC) (eWater Cooperative Research Centre, 2010b; eWater Cooperative Research Centre, 2010a) to predict concentrations on a 16 km stretch downstream of the discharge point of the WWTW SA2. The river itself is vital to the water supply of the city of Adelaide, supplying the Mount Bold and Happy Valley Reservoirs and the stretch receiving effluent from SA2 has been identified as a possible site for endocrine disruption by the South Australian Environmental Protection Agency (Goonan, 2008). Like LF2000-WQX, Source Catchments also provided a digitised river network, with a node-link system (Figure 2.4) representing a series of interconnected river stretches with artificial influences incorporated. However, in this case flow through the stretches (links) was calculated based on the SIMHYD rainfall-runoff model with Laurenson flow routing (eWater Cooperative Research Centre, 2010a). Whilst this uses historical data, it does not provide a flow distribution for each stretch. Instead it produces flow scenarios based on past events using historic data. As a result, in this study the model was run from 1/1/2008-31/12/2008, the year on which prescriptions data were based, to predict the daily concentrations on each stretch. Chemical input was simulated with an inflow function at the node representing SA2 based on a time series of daily concentrations modelled using the daily flow rates from the WWTW in 2008 to simulate a continuous influx. Another inflow function was incorporated at a node downstream representing the inter-basin transfer of raw River Murray water from the Murray Bridge-Onkaparinga pipeline by adding flow only as no WWTWs discharge within 500 km from this additional water source. The chemicals were transported through the interconnected stretches from the source with their concentrations calculated on each stretch based on the available dilution from simulated flows. A simple exponential decay model was incorporated for the steroid oestrogens only, which used half-lives based on their typical degradation rates in UK rivers at 20^{o}C water temperature (Jürgens *et al.*, 2002). These were 2.99 days for E1, 2.78 days for E2 and 17 days for the less degradable EE2. However, this was not temperature dependent and it should be recognized that their degradation could differ in Australian rivers due to different environmental conditions. However, no data are available to support this possibility. Like in LF2000-WQX no loss to sediment was assumed but in contrast, the conversion of E2 to E1 was not included, which could result in a small underestimation in concentrations of E1. In addition, the model did not incorporate the farm dam directly downstream of the WWTW which abstracts some water for irrigation, potentially affecting the concentrations of chemicals entering the main river stretch below this point particularly

during the summer months. However this could not be quantified. Once again, no removal at the WWTWs was assumed for the anti-androgens and no decay model was used to provide a worst case scenario.

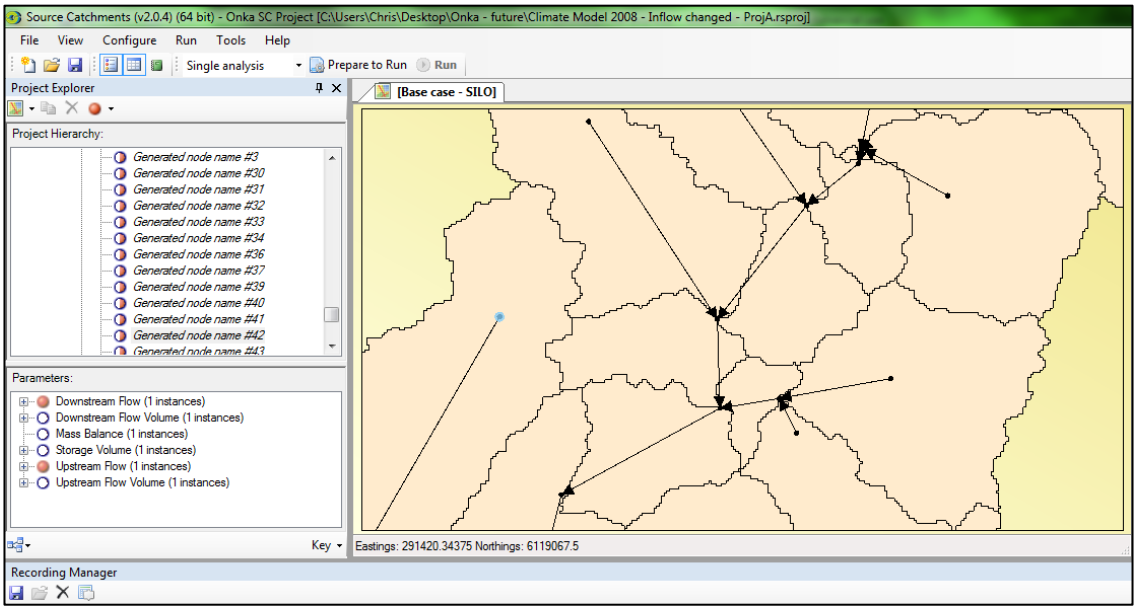


Figure 2.4 *Source Catchments software showing the link-node representation of part of the Onkaparinga River downstream of SA2.*

2.2.7 Risk assessment of the equivalent oestrogenic activity

Unlike the anti-androgenic pharmaceuticals, there is extensive data on the effects of steroid oestrogens *in vivo*, so their concentrations were compared with PNECs produced by the UK Environment Agency (Young *et al.*, 2004). Since oestrogens exist in the environment in combination and act additively to induce similar biological effects, it is appropriate that a combined "toxic equivalent" is incorporated into any risk assessment (Young *et al.*, 2004). This is presented as the E2 equivalent (EEQ) in ng/L which was calculated based on the following equation dividing the concentration of a steroid oestrogen by its PNEC:

$$\mathrm{EEQ} = \left(\frac{\mathrm{EE2\ (ng/L)}}{0.1}\right) + \left(\frac{\mathrm{E2\ (ng/L)}}{1}\right) + \left(\frac{\mathrm{E1\ (ng/L)}}{3}\right)$$

The PNECs were based on *in vivo* studies of the effects of these chemicals on fish and are discussed in Young *et al.* (2004) Briefly, for EE2, a PNEC of 0.1 ng/L was derived from a full life cycle study of zebrafish based on the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) for fertilisation success and incorporating a safety factor of five (Wenzel *et al.*, 2001). A PNEC of 1

ng/L was derived for E2 based on an early life study of Japanese medaka where 10 ng/L E2 produced an all-female population (Nimrod and Benson, 1998), incorporating a safety factor of 10. With less data available for E1, a PNEC of 3-5 ng/L was derived from studies of vitellogenin induction, such as Routledge et al., 1998. The more conservative PNEC of 3 ng/L was employed for estimating the EEQ in this study.

To determine the risk to wild fish, the hydrological models of the rivers were used to map potential "hot spots" for oestrogen concentrations. Stretches were categorised as "no risk", "at risk" or "high risk", based on the EEQ (<1, 1-10 and >10 ng/L EEQ respectively) (Williams *et al.*, 2009). Stretches were considered "at risk" if the EEQ exceeded 1 ng/L, based on the PNEC for E2. On these stretches, effects on individual fish, such as intersex, may be expected to occur. Additionally, stretches were identified as "high risk" when concentrations exceeded 10 ng/L EEQ, where effects on fish populations could be expected. This was based on *in vivo* evidence in which steroid oestrogens caused effects relevant to populations, which caused 100% feminisation of medaka exposed to 10 ng/L E2 and reduced fertilisation success at 1 ng/L EE2 (10 ng/L EEQ) (Schäfers *et al.*, 2007; Nimrod and Benson, 1998). This method of predicting the presence of "risk" stretches from the effluent model and LF2000-WQX has recently been compared with LC-MS/MS analysis on the Erewash, where modelled and measured concentrations both produced the same risk categories for the river stretches based on the EEQ (Williams *et al.*, 2012).

2.2.8 Predicting concentrations in 2050: the effects of population and climate change

2.2.8.1 Population projections

To determine how concentrations of these chemicals in effluents and rivers could change in the future, concentrations were modelled based on data relevant to 2050. These were then compared back to the predictions detailed above, produced from sources dating from 2001-2011, which are henceforth referred to as predictions for the present day. Data on population change was gathered from the "National Population Projections, 2010-based Projections" publication released in 2011 by the ONS, UK (Office for National Statistics,) and "Population Projections Australia, 2006-2101" released in 2008 by the ABS (Australian Bureau of Statistics, 2008) (Figure 2.5). Since projections were available for 2051 for both countries, these were assumed to be representative of 2050 and relevant to the local catchment areas. Three main

projections were used for each country based on demographic assumptions of future fertility, mortality and migration to produce different scenarios for population change. These included a principal projection (B) which assumed that current trends in these demographic assumptions would prevail in the future and high (A) and low (C) population projections to provide a range.

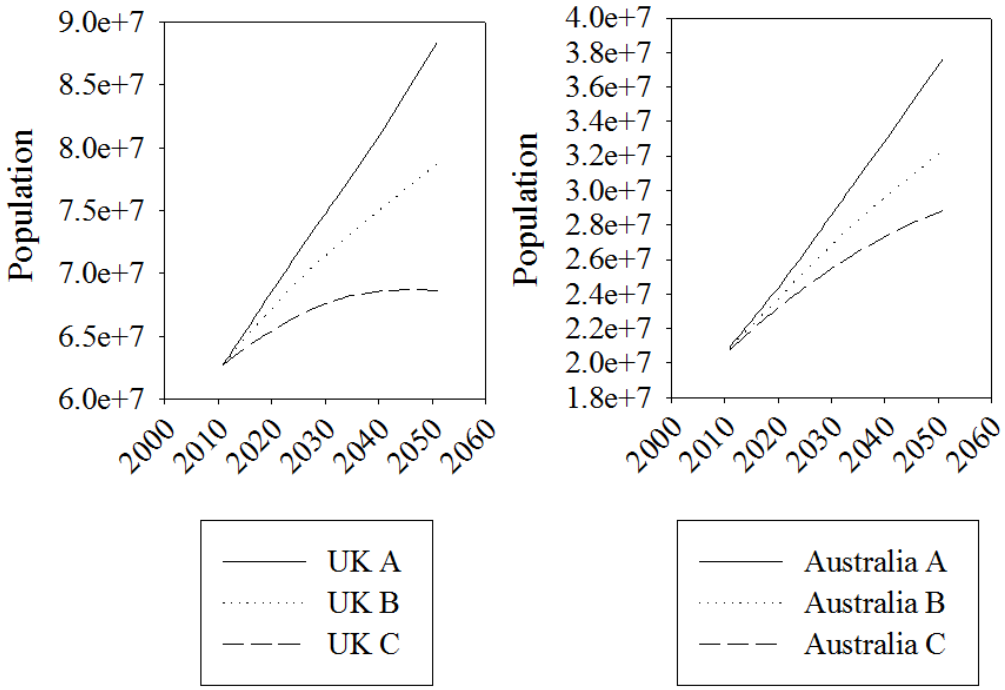


Figure 2.5 *The change in population to the year 2050 in the UK (left) and Australia (right) under the three population projections: A (high), B (principle) and C (low). Sourced from the Office for National Statistics, UK and the Australian Bureau of Statistics, Australia.*

2.2.8.2 Anti-androgenic pharmaceuticals

To predict the use of anti-androgenic pharmaceuticals in 2050, assumptions were made of the potential changes in the prevalence of the conditions for which they are prescribed. All four are used in the treatment of prostate cancer, although cyproterone acetate is also prescribed in other formulations to control male sexual desire and as part of the combined contraceptive pill with EE2. The incidence of prostate cancer by 2050 for each population projection was predicted based on records from England (1971-2010) (Office for National Statistics, 2013) and Australia (1982-2009) (Australian Institute of Health and Welfare, 2012) using the linear regression model (Figure 2.6).

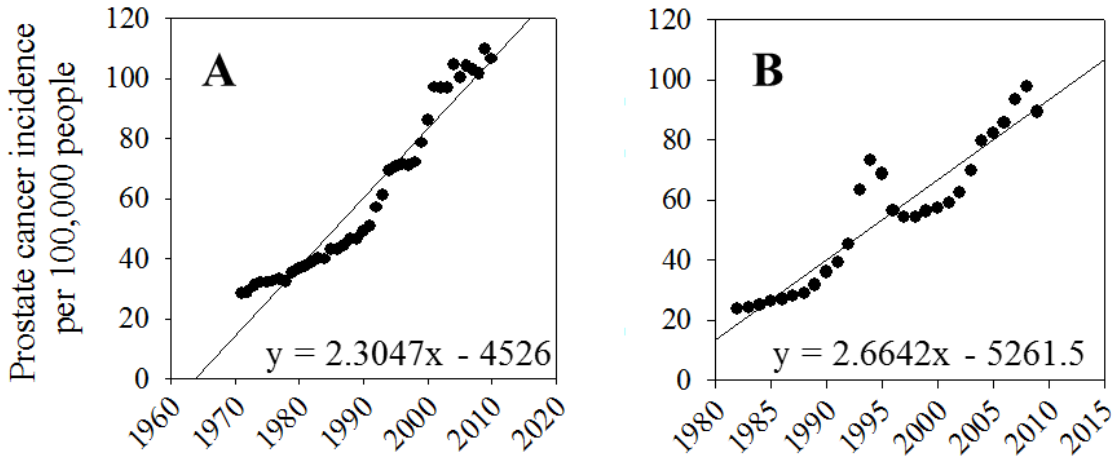


Figure 2.6 *Incidence of prostate cancer per 100,000 of the population in England (A), and Australia (B). Data from England used age-standardised rates standardised using the European Standard Population, whereas Australian data was estimated based on the annual incidence within the total population. The linear regression equation is shown.*

For each population projection, the prescriptions of each pharmaceutical used in the treatment of prostate cancer were increased in line with the projected rise in the disease. However, in the UK flutamide has been recently discontinued by the NHS so it was assumed that bicalutamide would be used as a replacement, since it has the same mechanism of action and the same use as flutamide. In this case, the increase in prescriptions of flutamide was converted to bicalutamide, based on three doses of 250 mg flutamide being equivalent to one dose of 50 mg bicalutamide. In Australia, the proportional use of the anti-androgens was assumed to stay the same. In the case of cyproterone acetate, the prescriptions of forms used in prostate cancer were also increased in line with the disease. However, in the UK data, those used in contraception were assumed to change in line with the proportion of menstrual females in each projection and those used to control male sexual desire were changed in line with the proportion of males over the age of 15 in the population (Table 2.5). In the Australian data, only prostate cancer and female contraceptive forms could be differentiated and their prescriptions were changed in the same way as the UK. The total population in each projection was then used alongside the original excretion data to produce new per capita loads. This method assumes that the age demographics of

the future population will remain constant and that the past trend will remain true to the future scenario.

2.2.8.3 Steroid oestrogens

The model for predicting the per capita load of steroid oestrogens is dependent on the composition of the population in terms of the different oestrogen excreting cohorts. Consequently, analysis of population projection data leading up to 2050 was completed to assess how they affected the per capita load and the total load of steroid oestrogens arriving at a WWTW. Since the projections data were available on an age by sex basis, new per capita loads for E1 and E2 were produced based on new oestrogen excreting cohorts relevant to 2050 to incorporate these changes in population composition (Table 2.5). Additionally, the per capita load of EE2 was changed in line with the proportion of menstrual females: the users of the contraceptive pill.

Table 2.5 *Cohort percentages based on census data from 2001 (UK), 2008 (Australia) and under the three population projections for 2050: high (A), principle (B) and low (C).*

UK

Cohort	2001	2050A	2050B	2050C
Menstrual females	23.5%	19.6%	19.9%	20.1%
Menopausal females	16.1%	18.7%	19.4%	20.3%
Menopausal females on HRT	1.3%	1.5%	1.6%	1.7%
Pregnant females	1.1%	1.3%	1.1%	1.0%
Males	39.0%	41.1%	41.6%	42.0%

Australia

Cohort	2008	2050A	2050B	2050C
Menstrual females	24.2%	21.4%	22.0%	21.3%
Menopausal females	13.7%	18.7%	18.7%	20.0%
Menopausal females on HRT	1.8%	2.5%	2.5%	2.7%
Pregnant females	1.3%	1.3%	1.2%	1.0%
Males	39.2%	43.7%	43.9%	44.5%

2.2.8.4 Predicting effluent concentrations for 2050

The effluent concentrations at the WWTWs under each population projection relevant to 2050 were calculated using the new per capita loads and assuming that the populations serviced would change in line with the population change from 2011-2051 (Table 2.6). No changes were made to the DWF at the WWTWs, which remained at the same volume as the present day to provide a worst case scenario by assuming that no additional water was available for dilution.

Table 2.6 *Fold changes in population from 2011-2050 applied to WWTWs in the UK and Australia based on the three population projections: A (High), B (principle) and C (low).*

	Population Change 2011-2050		
	2050A	2050B	2050C
UK	1.41	1.26	1.09
Australia	1.80	1.55	1.39

2.2.8.5 Climate change and river flow

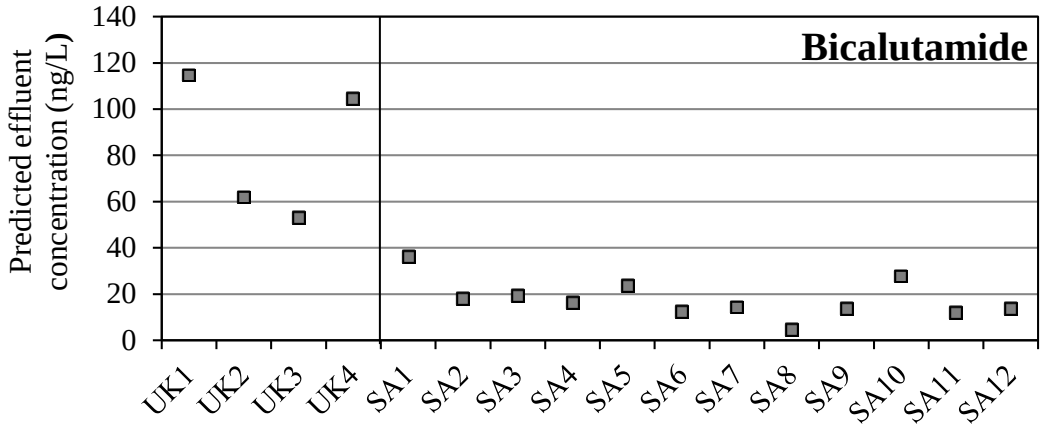
The river models used the new per capita loads at the WWTW inflows and were modified to incorporate predicted climate-induced changes to flow. In the UK, the flow on the Erewash in LF2000-WQX was modified with flow data from the UK Climate Projections (UKCP09) simulation *afgcx*, which is one of 11 physically plausible simulations relevant to a medium emissions scenario in the UK (Prudhomme *et al.*, 2012). As a result, the flows were on average 5.2% lower than the 2009 model on each stretch. Oestrogen concentrations along the river were again calculated with inflow from the WWTWs based on the updated population data relevant to each projection. Again, no changes were made to the DWF. Due to the lack of available data for South Australia, the Source Catchments model was modified by reducing flow on each stretch by 17.5% from its 2008 flow volume to provide a medium range climate model. This was based on a 15-25% reduction in annual stream flow for the Murray River projected for 2050 using two medium sensitivity climate scenarios, A1 and B1, from the Special Report on Emissions Scenarios (Beare and Heaney, 2002). It should be emphasised that these are average flow values, which do not present a scenario for episodic high flow events, which have been predicted under climate change scenarios, particularly for the UK (Bates *et al.*, 2008).

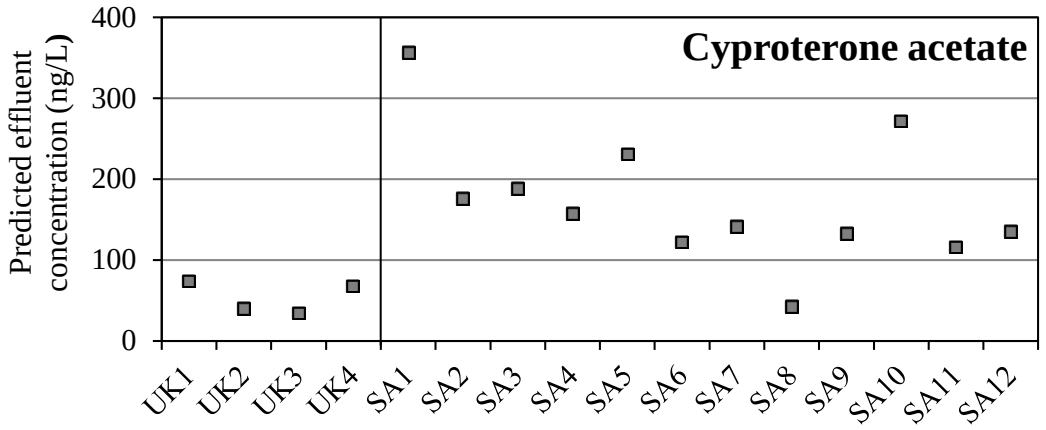
2.3 Results and discussion

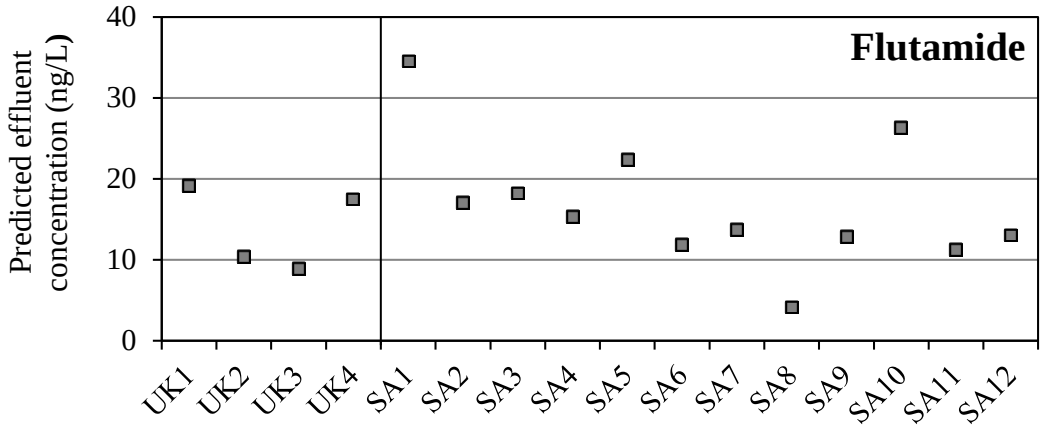
2.3.1 Predicted effluent concentrations in the present day

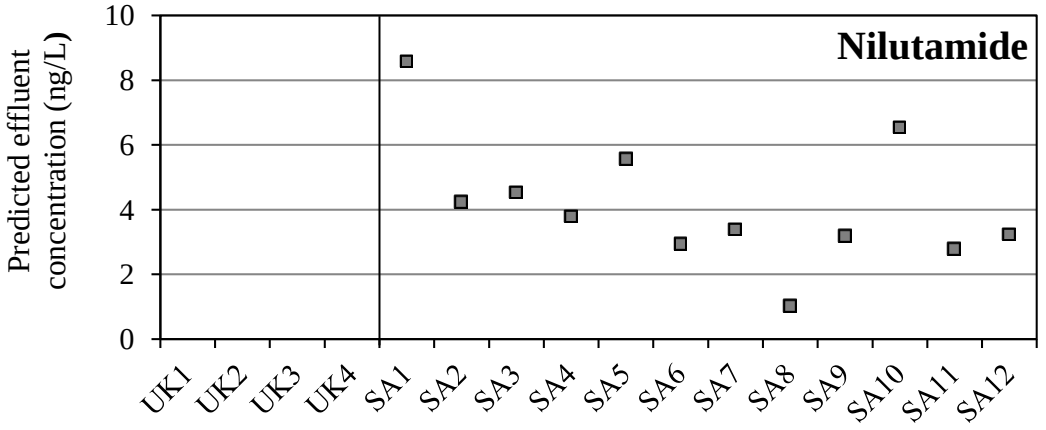
Concentrations of anti-androgenic pharmaceuticals and steroid oestrogens were modelled in UK and South Australian WWTW effluents based on annual prescriptions data. These produced a single concentration estimate for each WWTW, for which similar patterns were predicted in the order of concentrations at the WWTWs from highest to lowest for all the modelled chemicals. The deviations in concentrations resulted from the differing per capita flows, which demonstrated the importance of dilution in predicting concentrations at a given WWTW. South Australian WWTWs tended to experience a lower chemical load arriving for treatment since they generally served smaller populations than the UK WWTWs. When combined with the lower flow rate at most of these WWTWs, the dilution factor (the per capita flow) tended to be similar to UK WWTWs. The relationship between these factors is displayed in Table 2.3.

Since removal rates were not incorporated, the differences in concentrations of anti-androgenic pharmaceuticals between the two countries agreed with differences in their per capita loads (Table 2.1). In the UK this resulted in the highest concentrations being predicted for bicalutamide, which exceeded 100 ng/L at UK1 and 4 (Figure 2.7) with a range of 53-115 ng/L. However, concentrations in South Australian effluent were much lower, with a range of 4-36 ng/L. In contrast, cyproterone acetate concentrations were predicted to be much higher in South Australia, with a range of 42-356 ng/L in comparison to 34-74 ng/L in the UK. In addition, concentrations of flutamide were lower and showed a similar concentration range of 9-19 ng/L and 4-34 ng/L for the UK and South Australia respectively. In fact, unlike the UK, flutamide and bicalutamide were prescribed at relatively similar frequencies in Australia, which produced similar effluent concentrations. Nilutamide was only prescribed in Australia and was predicted to occur from 1-8.6 ng/L.


Bicalutamide
Predicted effluent concentration (ng/L)
0
20
40
60
80
100
120
140
UK1
UK2
UK3
UK4
SA1
SA2
SA3
SA4
SA5
SA6
SA7
SA8
SA9
SA10
SA11
SA12


Cyproterone acetate
Predicted effluent concentration (ng/L)
0
100
200
300
400
UK1
UK2
UK3
UK4
SA1
SA2
SA3
SA4
SA5
SA6
SA7
SA8
SA9
SA10
SA11
SA12


Flutamide
Predicted effluent concentration (ng/L)
0
10
20
30
40
UK1
UK2
UK3
UK4
SA1
SA2
SA3
SA4
SA5
SA6
SA7
SA8
SA9
SA10
SA11
SA12


Nilutamide
Predicted effluent concentration (ng/L)
0
2
4
6
8
10
UK1
UK2
UK3
UK4
SA1
SA2
SA3
SA4
SA5
SA6
SA7
SA8
SA9
SA10
SA11
SA12

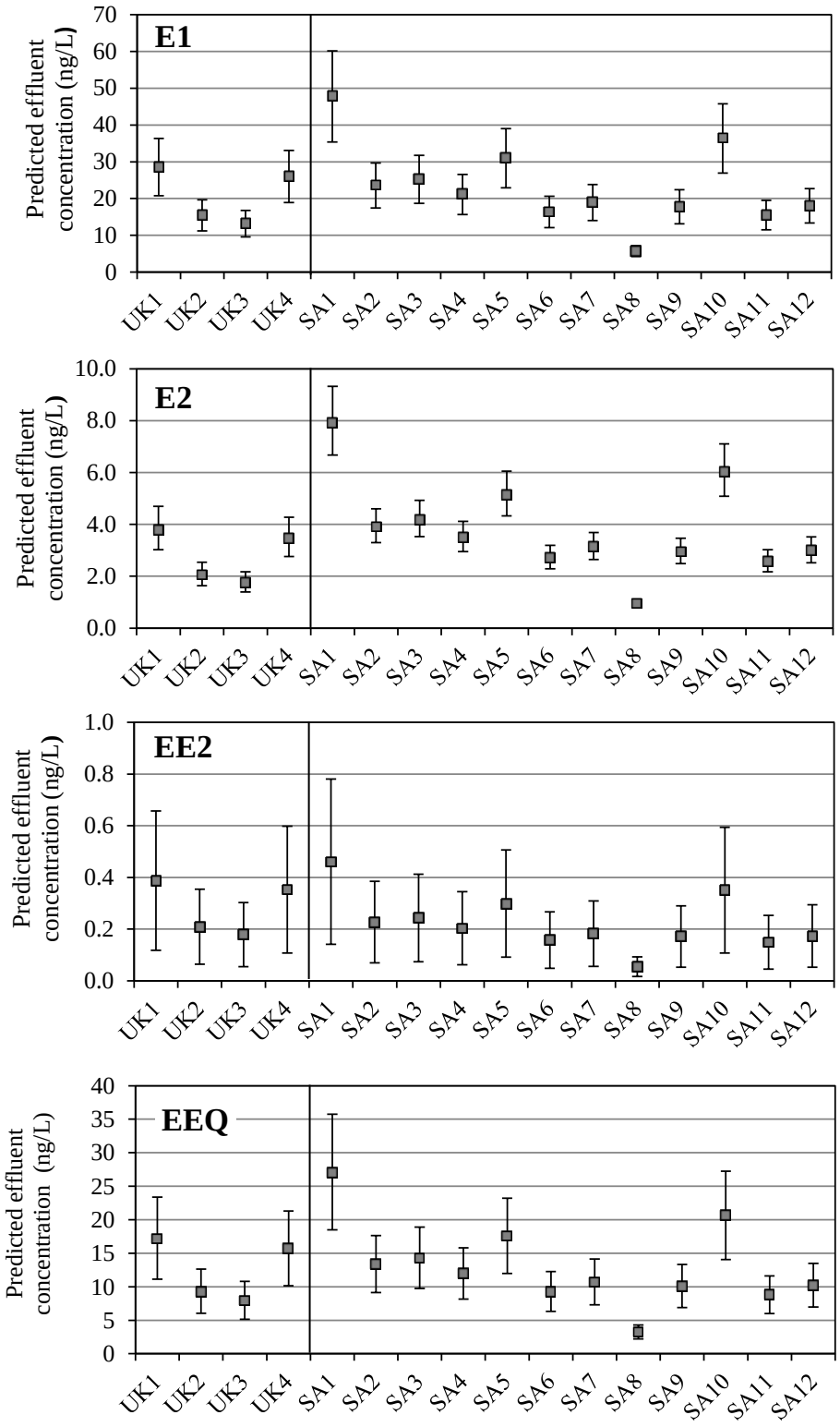


Figure 2.7 *The average predicted effluent concentrations of anti-androgenic pharmaceuticals and steroid oestrogens in the UK and South Australia. Boxes represent the predictions based on the average per capita loads (squares). Error bars for the steroid oestrogens extended to the predicted concentrations based on the upper and lower per capita loads for E1 and E2 and excretion rates for EE2.*

The steroid oestrogens were modelled based on population data for E1 and E2 and prescriptions data for EE2. Prescriptions of EE2 were also shown to differ between the two countries, which produced a higher per capita load in the UK. This differed from that of E1 and E2, where the differences in population demographics resulted in a higher per capita load in Australia (Table 2.1). However, due to the similar per capita flows at the WWTWs, the predicted concentrations of E1, E2, EE2 and the EEQ were similar in both the UK and South Australian effluents (Figure 2.7), corresponding with the measured data range from the two countries and a review of effluents globally (Allinson *et al.*, 2010). Based on the steroid oestrogen data, discharge from these WWTWs could be cause for concern for fish health if there is insufficient dilution available in rivers, based on the Environment Agency PNECs (Young *et al.*, 2004). In particular, SA1 showed very high concentrations as a result of its low per capita dilution and therefore warrants further investigation in the future.

2.3.2 The relevance to real world effluents

In previous studies, predictive modelling has been shown to produce environmentally relevant estimations for chemical pollutants originating from WWTWs (Johnson *et al.*, 2008). To assess the model, predicted concentrations of steroid oestrogens at UK2 were compared with measured concentrations in the UK. There were no measured data available for the anti-androgenic pharmaceuticals, although modelled data estimated that cyproterone acetate could occur at relatively comparable concentrations of 10-40 ng/L in WWTW effluent in France (Besse and Garric, 2009). In addition, concentrations of flutamide in the UK are now likely to be significantly lower than those predicted, if it is present at all, since the drug has been discontinued.

On a national scale the range of concentrations for the steroid oestrogens predicted by this study for both the UK and South Australia were within the range of measured concentrations from 43 UK WWTWs (Johnson *et al.*, 2007b) and over 70 WWTWs in Australia (Allinson *et al.*, 2010; Mispagel *et al.*, 2009; Ying *et al.*, 2009; Ying *et al.*, 2008; Tan *et al.*, 2007; Williams *et al.*, 2007; Leusch *et al.*, 2006; Braga *et al.*, 2005; Li *et al.*, 2004) (Table 2.7). The exception to this was SA1, which exceeded the 54 ng/L reported maximum observed concentration of E1 in Australia (Braga *et al.*, 2005). Although the range provided by the assessment of 70 effluents is relatively extensive, it only represents a small proportion of Australian WWTWs and it is plausible that higher concentrations could occasionally occur in some of the older WWTWs.

Table 2.7 *The mean average (upper-lower) oestrogen concentrations measured in effluents from 43 WWTWs in the UK and over 70 WWTWs in Australia based on the available literature. These are compared with the range of modelled concentrations in effluents from both countries.*

	Measured Concentrations (ng/L)				Modelled Concentrations (ng/L)			
	E1	E2	EE2	EEQ	E1	E2	EE2	EEQ
UK	<0.5-100	<1-22	<1-3.20	(11-87)	21 (10-36)	2.8 (0.6-4.7)	0.28 (0.05-0.66)	13 (5-23)
Australia	20 (0-54)	3.5 (0-18.5)	0.41 (0-1.3)	14 (0-50)	23 (4-60)	3.2 (0.6-7.9)	0.22 (0.02-0.78)	13 (2-36)

In a review of comparisons between modelled and measured data, predicted concentrations of pollutants in effluent were routinely predicted within a factor of 5 of the measured values (Johnson *et al.*, 2008). At UK2, when modelled concentrations were compared with measured concentrations from effluent samples collected between July and December 2009, clear temporal variation was observed in both datasets (Figure 2.8). The differences between measured and modelled concentrations at each sampling point also varied where predictions for E1 and E2 both tended to overestimate the measured by a factor of 0.9-54 (median 3.1) and 0.8-33 (median 4.7) respectively. However, modelled concentrations of EE2 tended to underestimate the measured by a factor of 0.2-1.5 (median 0.4). These deviations in opposing directions produced a smaller deviation in the modelled EEQ, which generally overestimated the measured by a factor of 0.5-3.0 (median 1.0). However, it is important to note that every WWTW is unique and that the deviations in the datasets observed at UK2 may be very different in another WWTW.

Based on the linear emission model these deviations cannot be explained by varying flow alone. Indeed, a lower actual per capita load and/or a higher removal rate could explain the overestimation of E1 and E2 and vice versa for EE2. At UK2, removal rates are reported to be higher than those assumed in the model for E1 and E2 (95 and 98% respectively) and lower for EE2 (32%) (Butwell *et al.*, 2010). When these measured removal values were input into the model, the deviation factor lowered to 0.2-9.4 (median 0.53) for E1, 0.1-3.8 (median 0.55) for E2, 0.7-6.1 (median 1.53) for EE2 and 0.5-3.0 (median 1.1) for the EEQ. This switched the original overestimation of E1, E2 and the EEQ and the underestimation of EE2, which suggests that the real removal rates are likely to be somewhere between the modelled and measured. The deviations

between the modelled and the measured data continued to vary across sampling points and are likely to be caused by variation in removal rates, which can cause 10 fold differences in day to day effluent concentrations (Williams *et al.*, 2003). Nonetheless, with these removal rates incorporated, all modelled data was within the measured range, demonstrating that a simple calibration of model parameters with data specific to a WWTW can improve the model performance. In particular, this could impact risk assessment, since different removal rates will affect both the proportions of oestrogens in effluent as well as their total concentrations, which will impact the EEQ. This also implies that river models will be more accurate with up to date removal data.

It could be argued that the deviation in the modelled concentrations in comparison to measured data negates the ability of such methods to provide an environmental risk assessment for oestrogens due to the low threshold values in the risk categories (Johnson *et al.*, 2008). Previous studies drawing comparisons have also found similar deviation, with oestrogen concentrations in point source discharges also deviating by an average factor of up to five (Johnson *et al.*, 2008). However, another study found that E2 was overestimated by a median factor of 10 in WWTW effluent (Williams *et al.*, 2012). Clearly, this model could not take into account the constant changes in the internal environment of an STW or the changing load, which are likely drivers of the fluctuating concentrations and the changing deviations between measured and modelled data. Indeed, variation in removal rates can cause 10 fold differences in day to day effluent concentrations (Williams *et al.*, 2003). From the river perspective, this deviation could make it difficult to adequately determine a risk category. However, in this study it should be considered that the effluent model was most accurate for the most potent oestrogen, EE2, which is critical in determining the EEQ. It also generally overestimated the EEQ, indicating that “at risk” river stretches downstream of the discharge were unlikely to be wrongly categorised as “no risk”. In fact, in a majority of cases the modelled concentrations fell within the range of the measured and if not then they exceeded it for all three compounds. When the updated removal rates were incorporated, all modelled data were within the measured range, demonstrating that a simple calibration of model parameters with data specific to a WWTW can improve the model performance. Consequently, the model should be considered to provide a good representative value for concentrations of compounds in WWTW effluents that are unlikely to underestimate risk categories downstream of the outfall. This basic effluent model is also supported by biological observations, where modelled steroid oestrogen concentrations at fish capture sites in the UK correlated well with intersex incidence and severity (Jobling *et al.*, 2006).

It is important to note that analytical measurements also have flaws that can affect risk categorisation. For example, deviation from the “real” concentrations can be achieved by degradation of the sample prior to extraction and analysis or through ion enhancement or suppression due to the complex nature of the sample. In addition, a measured concentration may not be representative if it is collected at the wrong time, due to the diurnal nature of concentrations of chemicals in WWTW effluent. There is also evidence for variation in the concentrations of chemicals in a single sample during inter-laboratory comparisons, which can have similar deviations to comparisons between measured and modelled data (Johnson *et al.*, 2008).

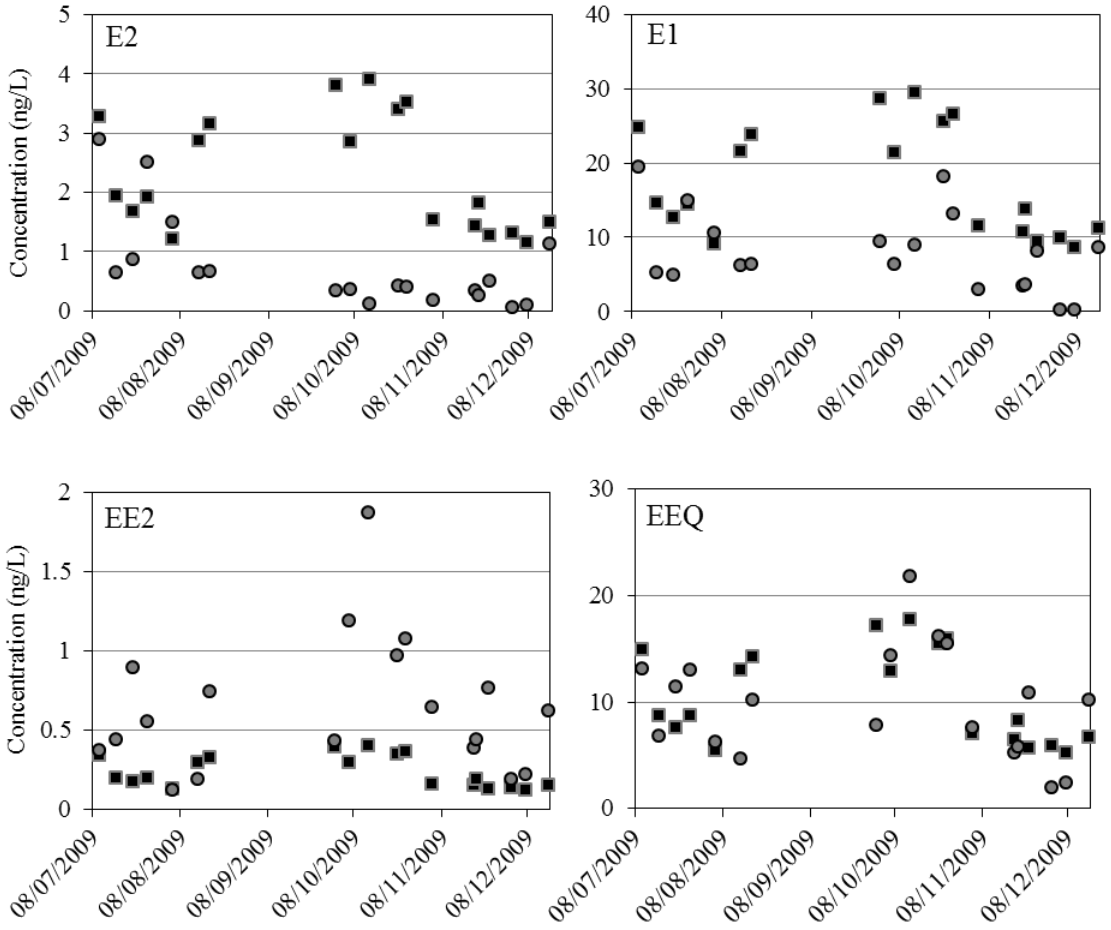


Figure 2.8 *The daily average modelled (squares) and measured (dots) oestrogen concentrations with the EEQ (ng/L) in effluent from UK2 over 19 sampling points from July to December 2009. A data gap exists between 27/8/09 and 21/9/09 due to the lack of available flow data to produce modelled concentrations.*

2.3.3 Predicted river concentrations

Predictive hydrological modelling of anti-androgenic pharmaceuticals and steroid oestrogens in the River Erewash, UK and the Onkaparinga River South Australia was completed based on their entry into the system via WWTW effluent input. The locations of the two rivers and a heat map of at risk areas are shown below in Figure 2.10, where the maps were produced in the geospatial processing program ArcMap 10 (Esri, USA).

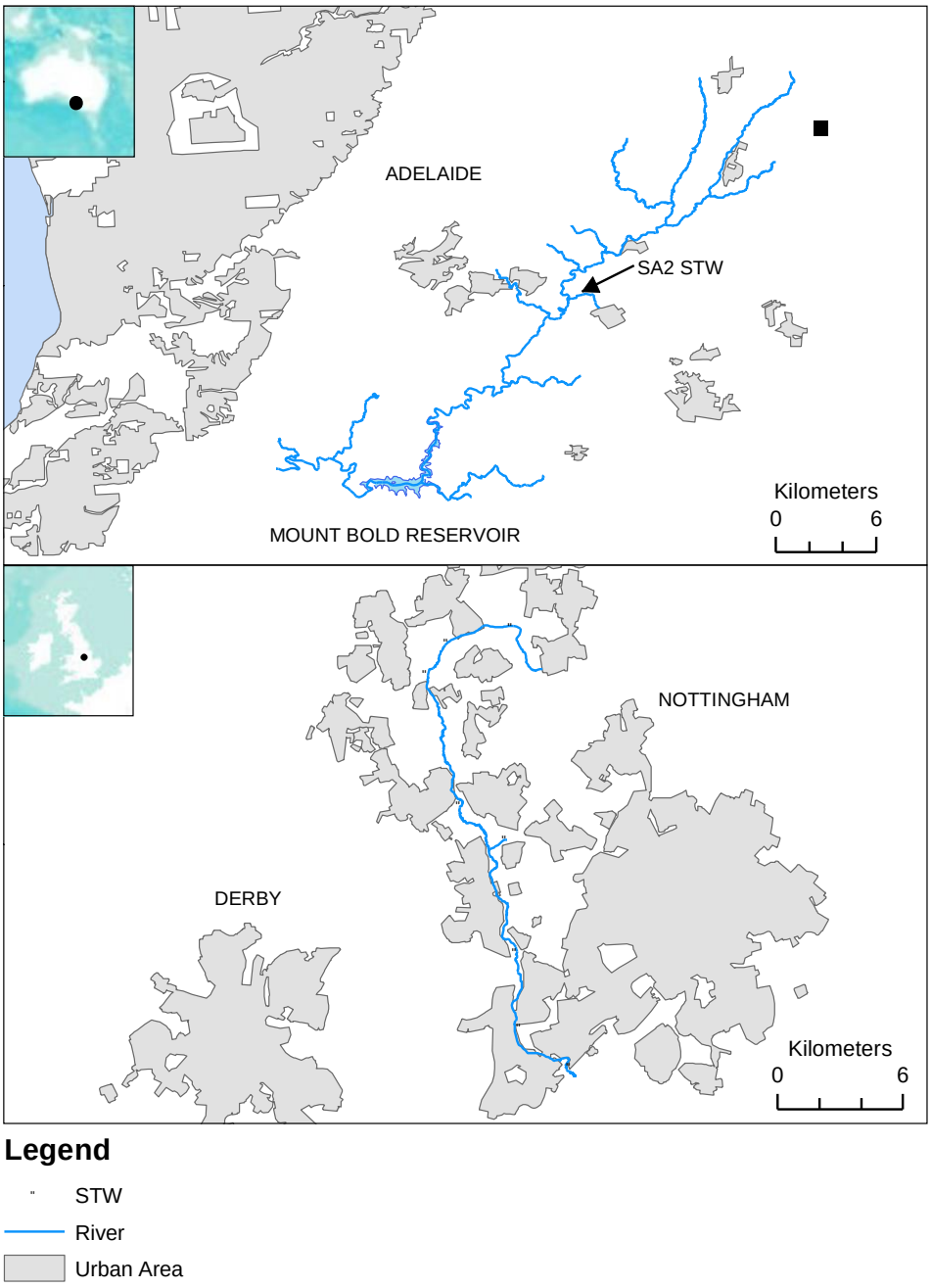


Figure 2.9 *Location maps showing the Onkaparinga River, South Australia (above) and the River Erewash, UK (below). Produced in ArcMap 10.*

2.3.3.1 Anti-androgenic pharmaceuticals

Hydrological modelling with LF2000-WQX found that the main river stretch of the Erewash was entirely contaminated with anti-androgenic pharmaceuticals, as a consequence of the assumption of constant influx from the eight WWTWs along the river (Figure 2.9). Accordingly, the upstream stretch and its tributaries remained unimpacted. Concentrations ranged dependent on the inflow from the WWTW and the available river dilution with an average of 26 (16-50) ng/L bicalutamide, 17 (10-32) ng/L cyproterone acetate and 4.3 (2.7-8.4) ng/L flutamide predicted on impacted stretches. On the Onkaparinga River, modelling with Source Catchments predicted high concentrations directly downstream of the single WWTW outfall, which reduced with dilution downstream along the entire 16 km stretch. Predicted concentrations on average were 4.3 (1.2-9.5) ng/L bicalutamide, 42 (12-94) ng/L, cyproterone acetate, 4.1 (1.2-9.1) ng/L flutamide and 0.1 (0.03-0.23) ng/L nilutamide.

2.3.3.2 Steroid oestrogen risk assessment

Hydrological modelling of the UK and South Australia rivers was used to identify potential hot spots of “at risk” areas for endocrine disruption in fish based on predicted concentrations of steroid oestrogens and their potential biological effects (Figure 2.10). On the River Erewash, UK, in agreement with data from the Johnson and Williams model (Williams *et al.*, 2012) almost the entire river was categorized as “at risk” of endocrine disruption in wild fish. The average EEQ was 2 (0-7) ng/L along the entire river, exceeding the 1 ng/L PNEC where effects on individuals such as intersex and vitellogenin induction are at risk of occurring (Williams *et al.*, 2009). This resulted from the assumption of constant influx from the eight WWTWs along the river which maintained the EEQ above 1 ng/L. On the Onkaparinga River in South Australia, concentrations were also predicted to exceed the 1 ng/L EEQ PNEC downstream of SA2. Around 9 km of the river was categorized as “at risk,” with concentrations decreasing with the distance downstream due to degradation and dilution from tributaries, eventually dropping below the PNEC upstream of the Mount Bold reservoir. An average EEQ of 3 (0.4-9) ng/L was predicted over these river stretches and individual steroid oestrogen concentrations were comparable with those measured at five river sites in Queensland at effluent outfalls and 1 km downstream of WWTWs (Ying *et al.*, 2009). They also compared with concentrations measured globally (reviewed by Ying *et al.*, 2002a).

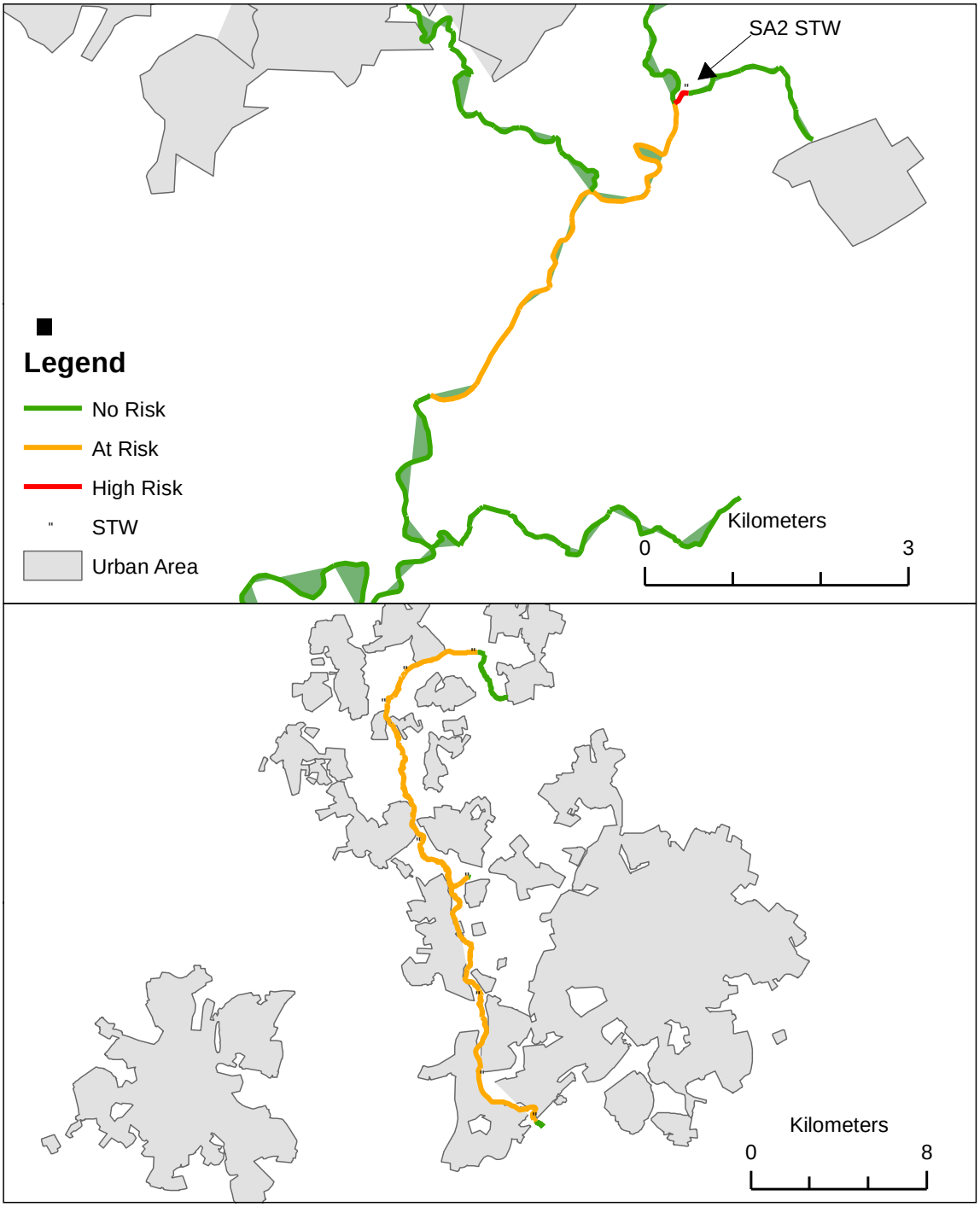


Figure 2.10 *Heat maps showing the risk categories of modelled stretches on the Onkaparinga River (above) and the River Erewash (below) in the present day based on the EEQ of the steroid oestrogens. Risk levels are indicated where "at risk" stretches exceed 1 ng/L and "high risk" stretches exceed 10 ng/L EEQ. Produced in ArcMap 10.*

2.3.4 Projected effluent concentrations by 2050

In both countries, the use of anti-androgenic pharmaceuticals and EE2 were predicted based on the projected population changes and assumptions made regarding how their use patterns could change by 2050. An assessment of the steroid oestrogens was also completed to determine how the changing population size and composition between the present day and 2050 would affect their concentrations in effluent. High (A), principal/medium (B) and low (C) projections were included for each chemical.

2.3.4.1 Anti-androgenic pharmaceuticals

The per capita load for each 2050 population projection was calculated based on the population change and the anticipated increase in prostate cancer incidence. In the UK, linear regression showed a 127-193% increase in the incidence of prostate cancer within the population projections A and C respectively in comparison to a 176-261% increase under their respective Australian projections. However, fluctuations were observed in prostate cancer incidence in the Australian data set between 1993 and 1995, which could reduce the accuracy of the projection (Figure 2.6). Nonetheless, this linear extrapolation method has been used by the Australian Institute for Health and Welfare for projecting future trends in a cancer incidence to the year 2020 to inform health service planning and future resource management (Australian Institute of Health and Welfare, 2012). However, the accuracy of these could be further improved in future studies by taking into account changes in population age demographics (Møller *et al.*, 2007).

Based on these data, prescriptions of these pharmaceuticals were all projected to rise, causing increases in their per capita load. The exception to this was flutamide, which was discontinued in the UK with the assumption that it would be replaced by bicalutamide. Bicalutamide increased to 32.2 and 7.8 µg/d in the UK and Australia respectively for all projections since prostate cancer incidence was assumed to be the same in each projection. Cyproterone acetate varied between projections due to its different uses, which also took into account the changing number of menstrual females in each projection (Table 2.6). The per capita load increased to 15.3, 15.4 and 15.5 µg/d for UK projections A, B and C and to 63.1, 64.0 and 57.5 µg/d under Australian projections A, B and C. Flutamide in Australia again had a similar per capita load to bicalutamide of 7.4 µg/d in each projection, whilst nilutamide remained low at 1.8 µg/d. The projected increase in per capita load, combined with increasing population served by each WWTW caused an increase in the concentrations of anti-androgenic

pharmaceuticals occurring in WWTW effluent in both countries in 2050 (Figure 2.11). This projected a greater chemical load available to enter the aquatic environment, with the worst case scenario in the high projection (A) as a result of its associated population growth. However, this assumes that the pharmaceuticals will remain in use at the same proportions as the present day, with the exception of flutamide, and that no alternative treatments become available. On the other hand, as generic and therefore cost effective drugs with a proven treatment history they are likely to remain popular for use in human medicine. Furthermore, alternatives may have similar mechanisms of action, which unless they degrade in an environmentally friendly fashion into inactive metabolites and transformation products, could still have similar impacts on fish health.

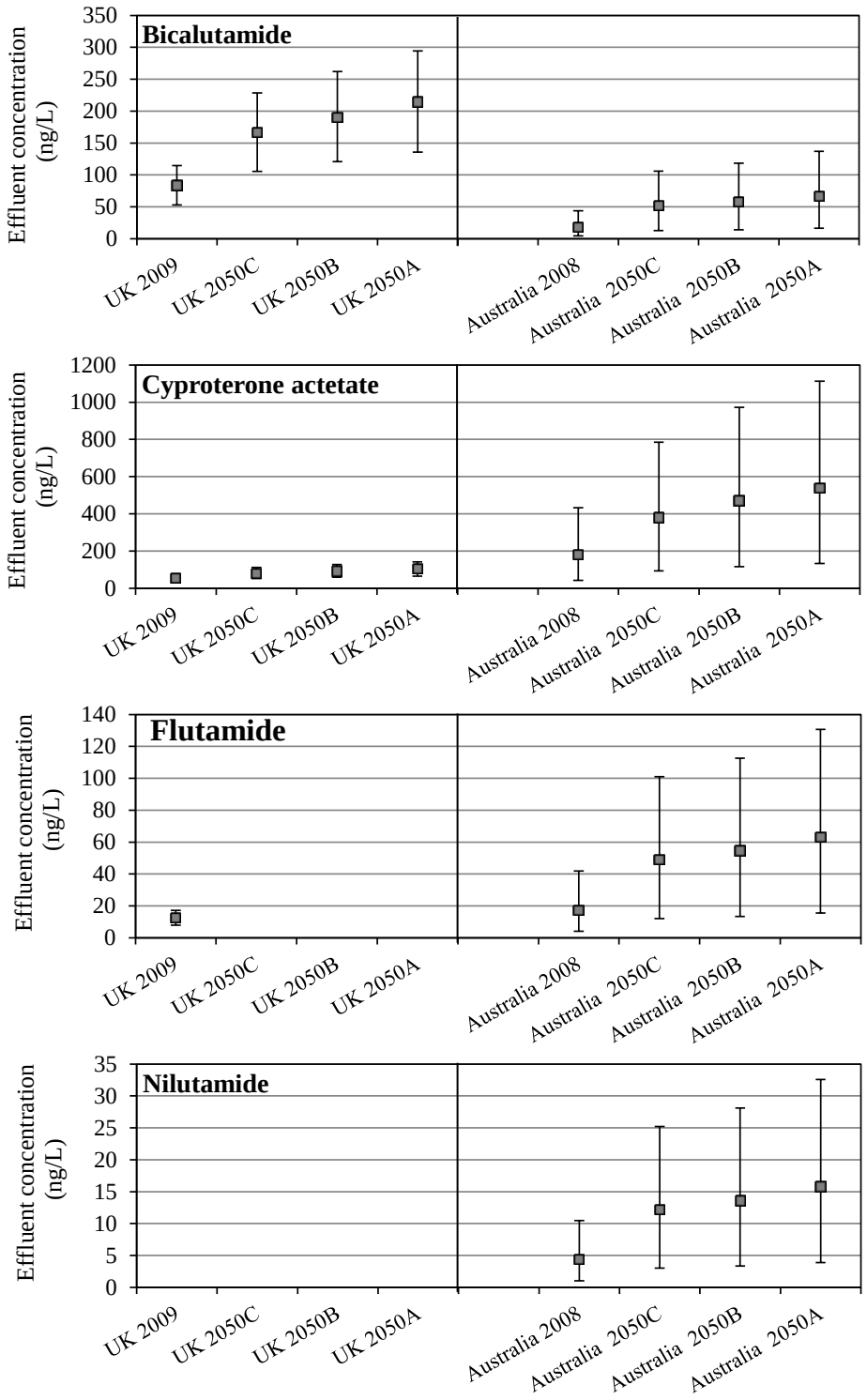


Figure 2.11 *The range of concentrations of anti-androgenic pharmaceuticals in effluents from UK and South Australian WWTWs under present day and future projections assuming no change in DWF. Boxes represent the mean (squares) with error bars extending to the minimum and maximum predicted concentrations from the four UK WWTWs and 12 Australian WWTWs. High projection (A), principle projection B) and low projection (C).*

2.3.4.2 Steroid oestrogens

Interestingly the change in population composition only had a small impact on the per capita load. A small increase occurred under the high projection and a small decrease occurred under the principle and low projections (Table 2.8), as a result of changes in the demographics of the female population. Specifically, whilst the proportion of higher oestrogen excreting cohorts, such as the menstrual females tended to decrease within the population, the lower oestrogen excreting groups, such as the menopausal females increased (Table 2.5). This caused a net reduction in per capita oestrogen load for the population. Interestingly, in the case of EE2 in the Australian scenario, the increase in the number of pregnant women in population projection A reduced the proportion of contraceptive pill users (menstrual females) relative to the other projections, causing a reduction in per capita load. Population growth had a much greater impact, resulting in an increase in the total oestrogen load arriving at the WWTW (Figure 2.12) and an increase in their subsequent concentrations in effluents to be discharged into the environment (Figure 2.13). The exception to this was the UK projection C, where effluent concentrations remained at similar levels since the increase in population was not sufficient to compensate for the lower per capita load. Again, the worst case scenario was observed with the high population projections, where effluent concentrations almost doubled by 2050 under the Australian projection A.

Table 2.8 *The per capita loads of the steroid oestrogens in µg/day for the present day and under the three population projections: A (high), B (principle) and C (low). These were used as the basis for effluent and river models.*

	Per capita load (µg/day)			
	Present Day	2050A	2050B	2050C
UK				
E1	14.02	14.66	13.76	12.78
E2	3.39	3.66	3.42	3.17
EE2	0.35	0.33	0.33	0.33
Australia				
E1	15.71	15.91	15.08	13.8
E2	3.9	4.08	3.85	3.55
EE2	0.28	0.27	0.27	0.27

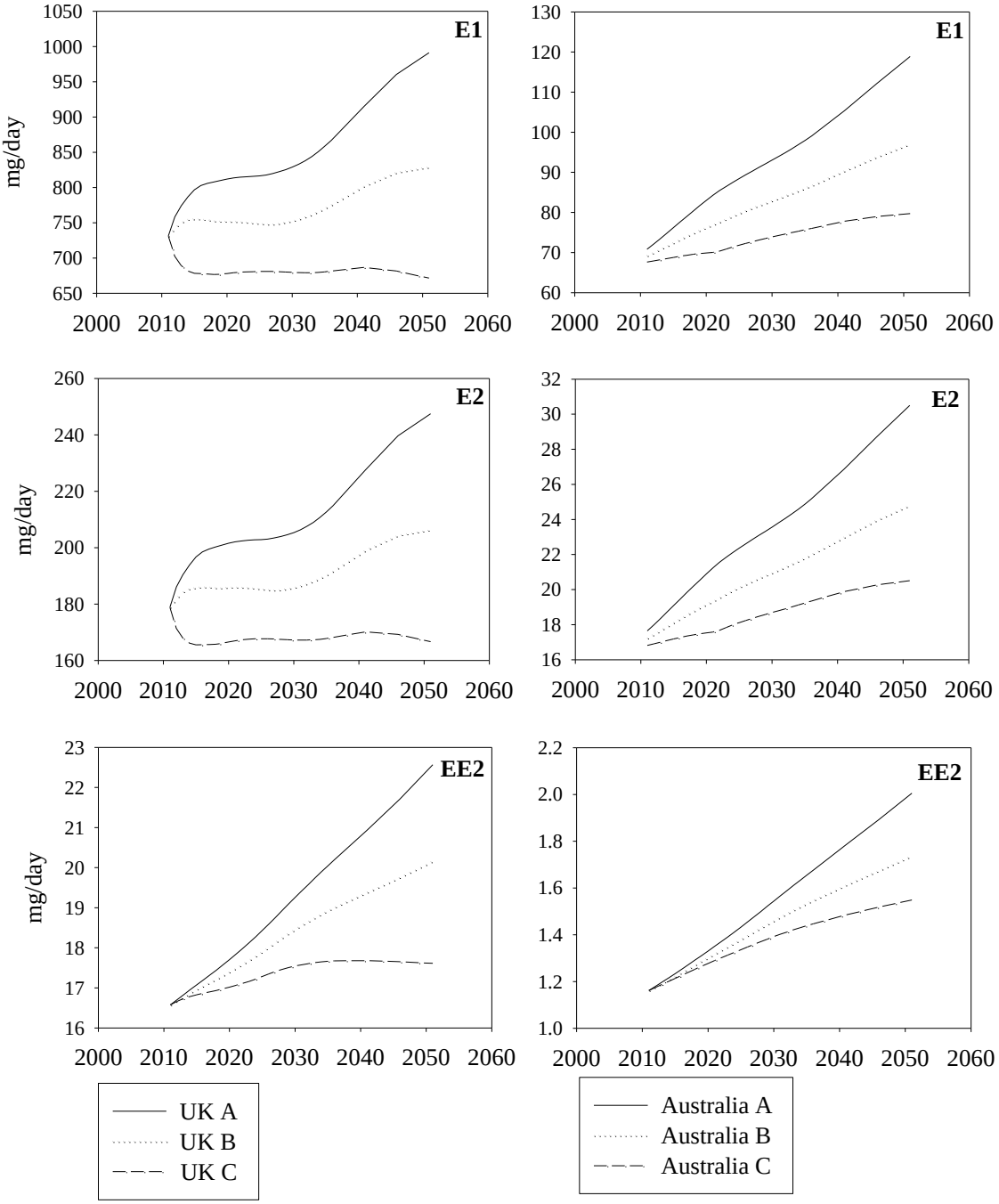


Figure 2.12 *The change in the total load (mg/day) of E1, E2 and EE2 arriving at WWTW's (UK2 and SA2) in the UK (left) and Australia (right) up to 2050 under the three population projections: High projection (A), principle projection B) and low projection (C).*

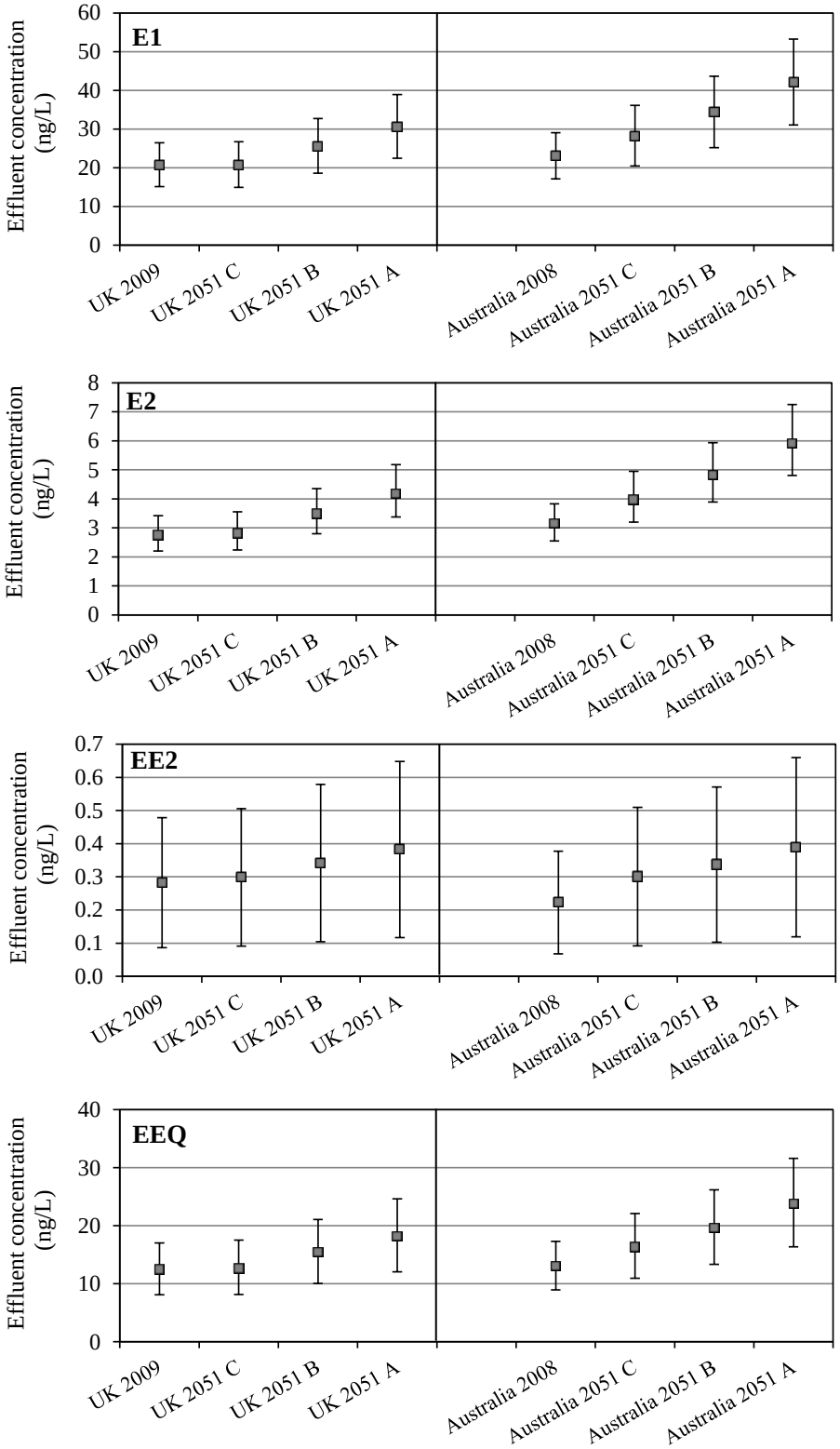


Figure 2.13 *The predicted steroid oestrogen and EEQ concentrations of effluents in the UK and South Australia under present day and future projections assuming no change in DWF at the WWTWs. Boxes represent the mean (squares) with error bars extending to the minimum and maximum concentrations from the four UK WWTWs and 12 Australian WWTWs.*

2.3.5 Projected river concentrations for 2050

The river models were modified for medium range climate scenarios with reduced flow and used in conjunction with the projected populations and their associated per capita loads to determine how river concentrations may change by 2050. In both catchments, decreased dilution in conjunction with increased chemical input caused increased concentrations of all modelled contaminants.

2.3.5.1 Anti-androgenic pharmaceuticals

On the River Erewash, although concentrations increased, there was no change in the number of impacted stretches from the present day. Bicalutamide experienced a 2-3 fold increase in its average concentration on impacted stretches from 26 (16-50) ng/L to 69 (42-135) ng/L in projection A, 62 (38-120) ng/L in projection B and 51 (32-100) ng/L. In comparison, cyproterone acetate only experienced a 1-2 fold increase from 17 (10-32) ng/L to 33 (20-64) ng/L, 30 (18-57) ng/L and 25 (15-48) ng/L in projections A, B and C respectively (Figure 2.14). On the Onkaparinga, the change in concentration was higher, increasing 3-4 fold for each projection, with the entire modelled section of the river impacted. Cyproterone acetate was again predicted to occur at the highest concentrations, increasing from 42 (12-94) ng/L to 168 (83-397), 145 (71-342) and 130 (64-307) ng/L. Bicalutamide increased from 4 (1-10) ng/L to 16 (4-49), 14 (3-42) and 12 (3-38) ng/L and a similar increase occurred with flutamide, from 4(1-9) ng/L to 15 (4-47) ng/L, 13 (3-40) and 12 (3-36) ng/L. In contrast, nilutamide still showed very low concentrations and increased from 0.1 (0.03-0.23) to 0.4 (0.1-1.2) in projection A and similar concentrations for projections B and C with an average of 0.3 (0.1-0.9) ng/L.

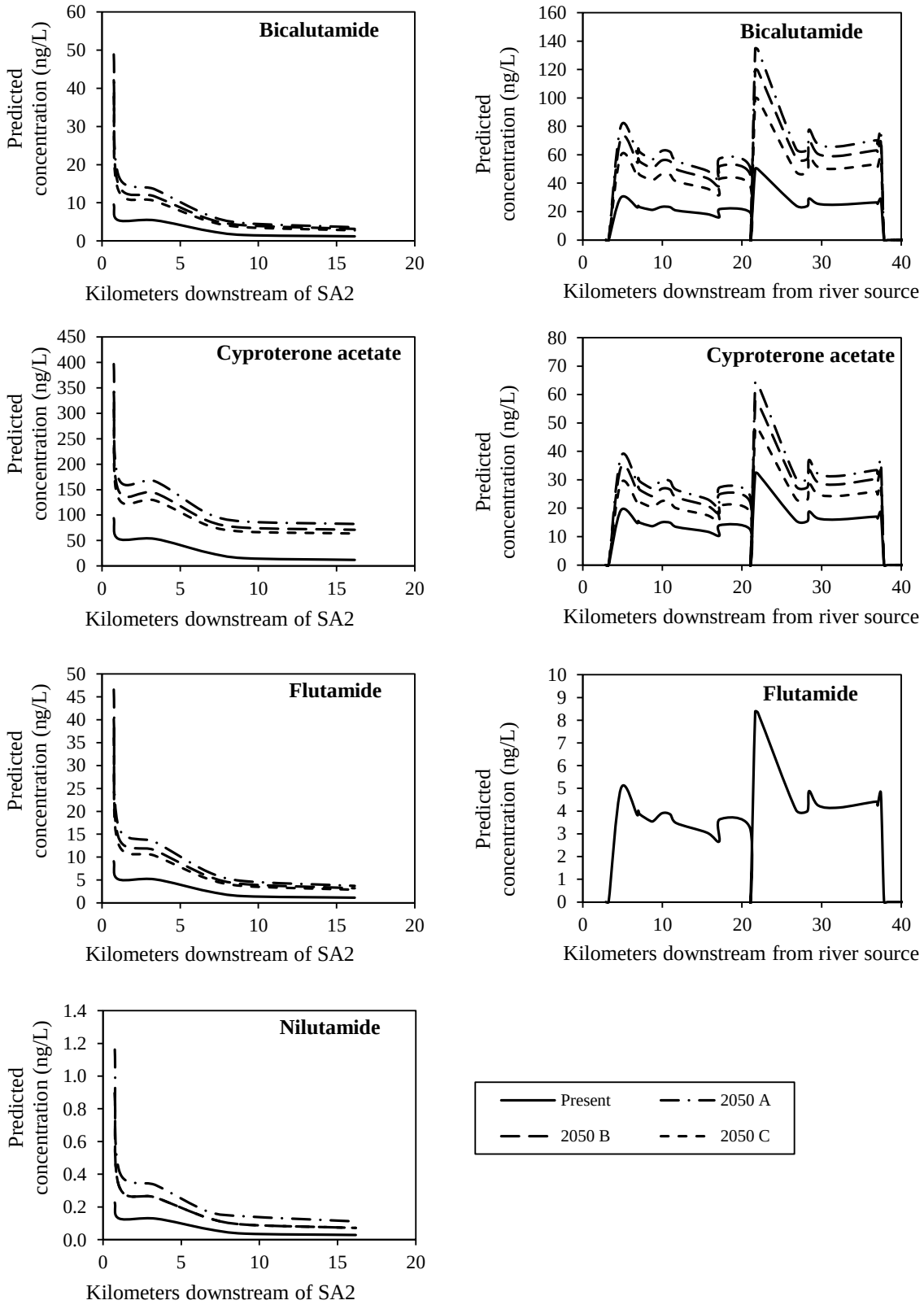


Figure 2.14 *Predicted river concentrations (ng/L) of anti-androgenic pharmaceuticals on the Onkaparinga River, South Australia (left) and the River Erewash, UK (right).*

2.3.5.2 Steroid oestrogens

The number of modelled stretches on the River Erewash predicted to be impacted and considered at risk (23/36) also did not change from the present day. However, in projection A, the EEQ increased to the point at which two stretches downstream of WWTWs became high risk. Under projections A and B increases in the average EEQ on impacted stretches from 3.7 (2.3-7.4) ng/L to 5.9 (3.6-11.6) and 4.9 (3-9.7) ng/L were predicted to occur respectively. However, in projection C the increase was smaller with an average EEQ on impacted stretches of 3.8 (2.3-7.5) ng/L due to the lower input of steroid oestrogens from the WWTWs. An increase in average EEQ was also predicted between the SA2 discharge and the Mount Bold reservoir on the Onkaparinga River under all population projections, from 2.9 (0.4-8.9) ng/L to 6.6 (1.8-18), 5.5 (1.5-15) and 4.6 (1.2-12) ng/L EEQ for projections A, B and C respectively. Importantly, the length of river downstream of the WWTW considered “at risk” increased under all three projections to include the entire 16 km modelled stretch upstream of the reservoir, whilst in projections A and B the stretch immediately downstream of SA2 became “high risk” (Figure 2.15).

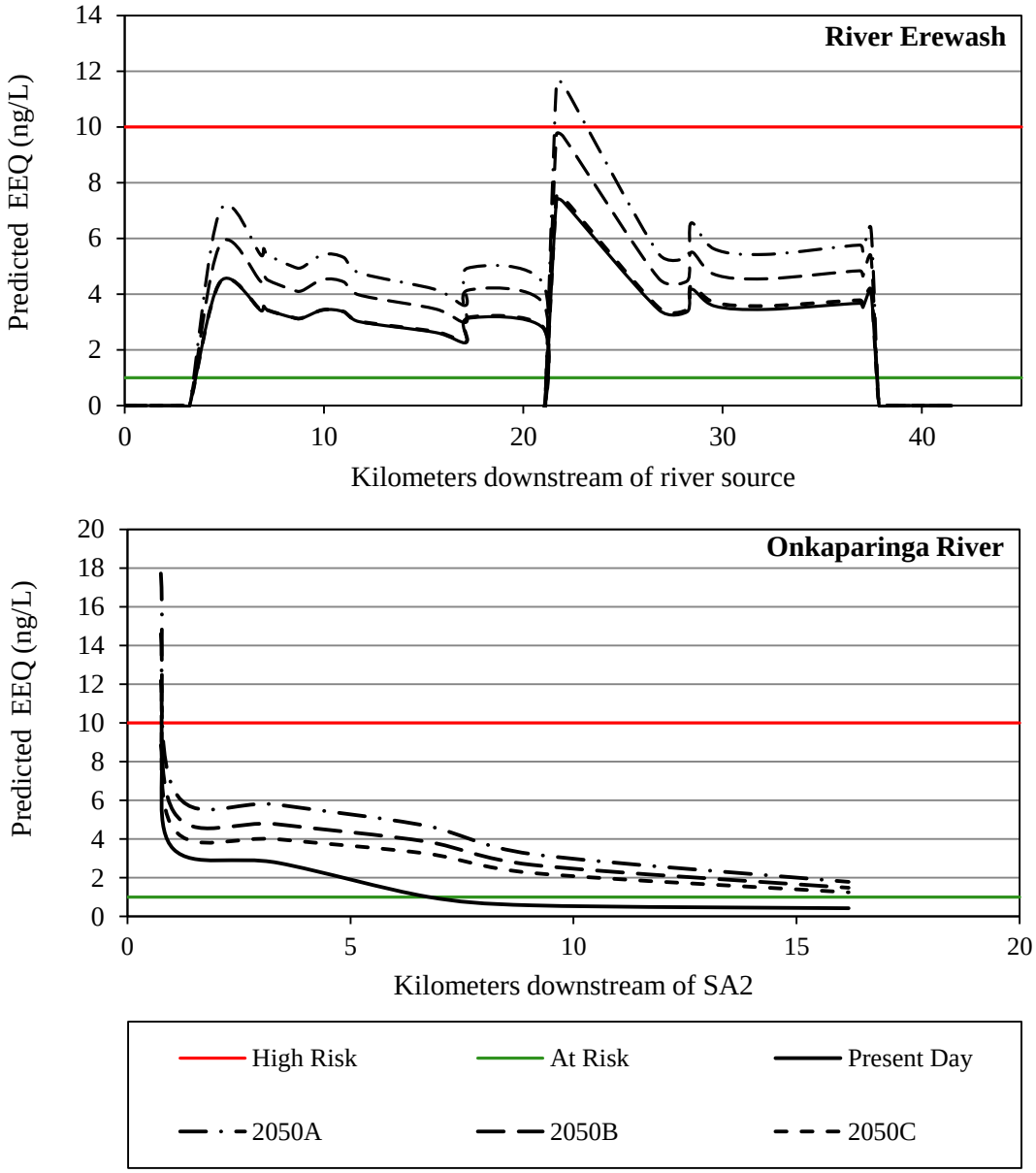


Figure 2.15 *The average predicted EEQs (ng/L) for the present day compared with the three future population projections, high (2050A), principal (2050B) and low (2050C), with river flows reduced for medium range climate change scenarios. Risk levels are indicated where "at risk" stretches exceed 1 ng/L and "high risk" stretches exceed 10 ng/L EEQ.*

Mitigation to combat these rising oestrogen concentrations may be achieved with increased removal efficiency at WWTWs with improved uptake of modern treatment technologies, many of which are already used for treating drinking water and recycled wastewater. This has already been demonstrated in the UK (Baynes *et al.*, 2012;

Butwell *et al.*, 2010; Johnson *et al.*, 2007a) and similar results have been found in Australia (Kumar *et al.*, 2012; Reitsema *et al.*, 2010). Indeed, in Western Australia the induction of the estrogenic biomarker vitellogenin was found in male fish downstream of a secondary treated rural effluent but not downstream of tertiary treatment (Reitsema *et al.*, 2010). However, a number of studies have also detected steroid oestrogen concentrations which exceed the PNECs upstream of WWTWs, demonstrating the importance of considering multiple origins of environmental steroid oestrogens (Reitsema *et al.*, 2010; Ying *et al.*, 2009; Williams *et al.*, 2007), such as agricultural runoff (Matthiessen *et al.*, 2006), as well as WWTW effluent. In addition, other chemicals with the potential to cause feminizing effects in wildlife, such as the alkylphenols, which have been restricted under EU legislation, are still in use in Australia and have been detected in surface water (Ying *et al.*, 2009).

It is important to note that there is still limited research on endocrine disruption caused by steroid oestrogens in native Australian fish species, although some have been shown to be susceptible under laboratory conditions (Woods and Kumar, 2011; Codi King *et al.*, 2008). Indeed, study of the Murray rainbowfish indicated a LOEC of 10 ng/L for E2 and 5 ng/L for EE2 in the induction of vitellogenin, which compares well with other laboratory fish models such as fathead minnows, zebrafish and Japanese medaka (Woods and Kumar, 2011). The effects on vitellogenin mRNA were also assessed in this study and compare well with a comparative exposure of laboratory model species and wild fish species from the UK, including roach, three-spined stickleback and rainbow trout (Lange *et al.*, 2012b). As a result, further investigation is warranted to determine how susceptible Australian species are to oestrogens from all sources. This should include laboratory exposures to chemical standards as well as WWTW effluents, although this should not be limited only to tertiary treated effluents. Indeed, by increasing the available data Australian PNECs could be derived that accurately reflect the risks and mitigation required to protect Australian biota.

2.3.6 Predicted concentrations of anti-androgenic pharmaceuticals in present and future scenarios and effects on fish

The anti-androgenic pharmaceuticals, with the possible exception of flutamide, have not been researched as extensively as oestrogenic contaminants and there is little knowledge of their potential effects at environmental concentrations. Nonetheless, the literature so far suggests that they exert adverse effects in fish in the µg/L range. In the case of bicalutamide, the single study published is a multigenerational study using

fathead minnows, which observed reductions in secondary sexual characteristics in the adults and impacted survival and reproduction in the F1 at 100 µg/L but not at 10 µg/L (Panter *et al.*, 2012). On the other hand, flutamide has been more extensively studied and has shown effects at lower concentrations with a LOEC from the literature of 2 µg/L, which caused testicular alterations in carp (Bottero *et al.*, 2005). Other studies of flutamide have not tested concentrations this low, but effects on sex steroids, weight, condition factor and fecundity have been observed at the lowest tested concentrations between 32 and 67 µg/L (Rajakumar *et al.*, 2012; Chakrabarty *et al.*, 2012; León *et al.*, 2007; Jensen *et al.*, 2004). There have been no studies of the effects of nilutamide on fish, but given its structural similarities to bicalutamide and flutamide it could cause effects at similar concentrations. In comparison, cyproterone acetate has been shown to cause adverse effects at lower concentrations than the other pharmaceuticals, inducing intersex at the lowest tested concentration of 1 µg/L in Japanese medaka (Kiparissis *et al.*, 2003). Consequently, the very low concentrations predicted to occur in both countries may suggest that an impact on fish is unlikely. However, there is evidence of effects of cyproterone acetate on sex steroid concentrations in mummichog at 1-100 ng/L, within the range of the predicted concentrations for the modelled effluents and rivers in both countries (Sharpe *et al.*, 2004). As a result, this drug presents an interesting topic for future research in both singular and mixture scenarios with other significant environmental EDCs known to co-occur with this chemical.

2.3.7 Considering multiple stressors

The projected concentrations for 2050 are based purely on population growth and changes in dilution, making no additional assumptions to how changing environmental variables may affect the availability of contaminants. For example, in the case of pesticides, increasing temperature can reduce their persistence (Bailey, 2004), whilst rising salinity can increase bioavailability by the “salting out” effect, where binding of water molecules to salts makes them unavailable for the dissolution of organic chemicals (Noyes *et al.*, 2009; Schwarzenbach *et al.*, 2005). In addition, the work does not make any assumptions regarding the effects of multiple stressors on fish, in which a combination of pollutants and adverse environmental conditions can have antagonistic, additive or even synergistic effects (reviewed in Holmstrup *et al.*, 2010). Indeed, environmental variables can affect the susceptibility of organisms to chemical stressors by changing the toxicokinectics and dynamics of pollutants. For example, the toxicity of the organophosphate insecticide phorate was significantly higher in saline conditions due to enhanced metabolism of the chemical to toxic metabolites (Lavado *et*

al., 2011). Furthermore, pollutants affect the ability of organisms to cope with external change, which was observed during exposure of larval mummichog to atrazine at different salinities where their ability to osmoregulate collapsed (Fortin *et al.*, 2008). These findings are summarised in a review by Hooper *et al.* (2013) who stated that: *in simplest terms, GCC [global climate change] can make organisms more sensitive to chemical stressors, while alternatively, exposure to chemicals can make organisms more sensitive to GCC stressors* (Hooper *et al.*, 2013).

In fish, adverse effects on reproduction and sexual development can be induced by changes to the ambient environmental conditions as well as chemical interference, particularly in the case of temperature change. Indeed, research suggests that temperature induced masculinisation is mediated by cortisol, which appears to reduce the production of hormones essential for ovarian development in vertebrates (Hayashi *et al.*, 2010; Carragher and Sumpter, 1990). In addition, experiments conducted with fathead minnows found that the rearing temperature could affect secondary sexual characteristics in males and females, which were reduced outside of the ambient temperature. The investment of energy between somatic and gonadal growth also appears to differ at high and low temperatures, with fish in warmer conditions capable of reproducing at a younger age (Brian *et al.*, 2011). Since fathead minnows are a thermo-tolerant species, this may suggest that they can respond to different environmental conditions to maximise their fitness. However, other species are not so thermo tolerant and globally changing environmental conditions could affect the abundance and distribution of fish species (Hooper *et al.*, 2013). Indeed, shifts in community structure on the Upper Rhônhe River have already be observed as temperature has increased over the last few decades and more thermophillic fish have progressively replaced cold water species (Daufresne *et al.*, 2004). Even though EDCs and environmental stress can affect these parameters, there has been little knowledge of how climate change could impact the effects of steroid oestrogens in fish. However, studies have shown that hypoxia has no effect on oestrogen induced vitellogenin induction in fathead minnows, whilst higher temperatures were shown to speed up this response, although the magnitude did not change (Brian *et al.*, 2011; Brian *et al.*, 2005). Nonetheless, there is abundant evidence to suggest that sexual disruption by steroid oestrogens occurs in a dose dependant manner, supporting that suggestion that increasing concentrations in the future will increase the incidence and severity of intersex (Jobling *et al.*, 2006; Metcalfe *et al.*, 2001). This was also demonstrated on the intersex endpoint during the steroid oestrogen exposures in this study.

2.3.8 Conclusions

This study demonstrated the use of predictive modelling as a tool for assessing potential EDCs originating from WWTW effluent in UK and South Australian catchments. This was the first time that these techniques had been applied to an Australian catchment. The results predicted that effluent discharge could cause concentrations of anti-androgenic pharmaceuticals to occur in river catchments in ng/L in both countries. However, with the exception of cyproterone acetate, the predicted concentrations are below those which have been shown to cause adverse effects in fish. The steroid oestrogens were modelled alongside these drugs in an assessment of the presence of river stretches at risk of endocrine disruptive effects occurring in fish. This demonstrated that the use of modelling techniques used in risk assessment in the UK could also be applied to Australian scenarios. Concentrations of steroid oestrogens in rivers in both countries were expected to exceed the 1 ng/L EEQ PNEC, implying that there is a risk of endocrine disruptive effects occurring in wild fish. This supports evidence in Australia of the feminization of non-native fish in effluent contaminated areas (Reitsema *et al.*, 2010; Rawson *et al.*, 2008; Batty and Lim, 1999). Furthermore, in the absence of mitigation strategies we anticipate an increase in the concentrations of environmental contaminants with human origins in UK and Australian river catchments by 2050. This was calculated based on population growth and reductions in river flow through climate change driving increases in environmental concentrations. Indeed, changes in these factors are expected to increase further by 2100 and beyond, so it is quite plausible that the magnitude of the change in concentrations of environmental contaminants will also increase further. However, it is important to note that additional variables may effect these predictions. For example, measures to conserve water may further reduce dilution of potential contaminants arriving at WWTWs, whilst increasing anthropogenic control of river flow and the use of recycled wastewater could result in additional changes to their dilution in rivers. In addition, an increasing occurrence of extreme weather events could cause greater changes in flow which could have more dramatic implications for concentrations than our modelling suggests. Indeed, variation in flow and dilution may be a much greater driver than population change alone, causing increases or decreases in concentrations that may differ from our model, depending on water availability. Since a better understanding of the drivers that cause at risk areas has been called for (Boxall *et al.*, 2012), these scenarios may provide interesting subjects for more detailed assessment in the future. Overall, the prediction of increasing concentrations of these contaminants suggests that endocrine disruption in wild fish may be a long-term management issue for which effective investment in pre-emptive mitigation today may pay off in the future.

CHAPTER 3: ASSESSING SEXUAL DISRUPTION BY ANTI-ANDROGENIC PHARMACEUTICALS IN FISH MODELS

3.1 Introduction

Anti-androgenic activity has been detected in environmental samples globally and commonly occurs in UK effluents, causing activity to be detected in downstream river stretches (Ma *et al.*, 2013; Grover *et al.*, 2011; Zhao *et al.*, 2011; Johnson *et al.*, 2007b; Urbatzka *et al.*, 2007). The impacts of this activity on fish health are of increasing interest, since anti-androgenic chemicals can disrupt the androgen signalling pathway of an organism (Hotchkiss *et al.*, 2008; Kelce and Wilson, 1997). By reducing the availability and function of androgens at the receptor site, anti-androgens demasculinise male fish under laboratory conditions, inducing traits associated with the widespread sexual disruption observed in wild fish populations. These include intersex, altered gene expression, and reductions in secondary sexual characteristics, sperm counts and fecundity (Filby *et al.*, 2007b; Jobling *et al.*, 2006; Ankley *et al.*, 2004; Jensen *et al.*, 2004; Kiparissis *et al.*, 2003; Bayley *et al.*, 2002). Furthermore, anti-androgenic effects on the androgen-dependent protein, spiggin, in wild stickleback (*Gasterosteus aculeatus*) have been observed downstream of a wastewater treatment works (WWTWs) effluent outfall (Katsiadaki *et al.*, 2012) and anti-androgenic activity has been detected in the bile of effluent-exposed fish (Rostkowski *et al.*, 2011; Hill *et al.*, 2010; Kinani *et al.*, 2010). However, the chemicals responsible for this activity in the environment remain largely uncharacterised.

Pharmaceutical anti-androgens seem likely candidates as potential contributors to anti-androgenic sexual disruption in wild fish. Indeed, of the oestrogenic contaminants detected in the environment, it is the steroid oestrogens which are the most potent and these also have a natural or pharmaceutical origin from human excretion. From this perspective, in chapter two, concentrations of anti-androgenic pharmaceuticals were modelled in WWTWs effluents and river catchments in the UK and South Australia as part of a targeted approach to identifying likely environmental anti-androgens of concern. This found bicalutamide and cyproterone acetate to be the two dominant anti-androgenic pharmaceuticals in both countries with concentrations occurring in ng/L. For example, along the River Erewash, UK, 26 (16-50) ng/L bicalutamide and 17 (10-32) ng/L cyproterone acetate were predicted to occur, in comparison to 4.3 (2.7-8.4) ng/L flutamide on impacted stretches. Aside from this study, the likely environmental concentrations of these pharmaceuticals have been poorly documented and there have been few studies of their effects on fish. Consequently, it is important to determine what contribution these chemicals could make to sexual disruption observed in wild fish, particularly in the context of mixture effects with other contaminants with similar modes of action.

3.1.1 Anti-androgenic pharmaceuticals and sexual disruption

Bicalutamide and cyproterone acetate have similar modes of action and are used in human medicine to treat androgen dependent disorders. Bicalutamide is a pure androgen receptor (AR) antagonist, in that it has no other receptor activity and specifically blocks the binding of circulating androgens to the AR. This in turn impacts androgen signalling within the HPG axis by blocking the negative feedback of circulating testosterone on ARs in the brain. This mimics androgen deficiency and causes an increase in the release of gonadotropin releasing hormone, which stimulates luteinising hormone secretion by the pituitary and causes a positive feedback on sex steroid production (Eri *et al.*, 1995). Although this increases circulating concentrations of androgens, their action at receptor targets remains blocked. However, increasing concentrations of circulating oestrogens can result in demasculinising side effects such as gynaecomastia, although spermatogenesis and libido is still maintained, at least to a certain degree (Neumann and Kalmus, 1991). In contrast, whilst cyproterone acetate is an AR antagonist, it also targets androgen synthesis through its anti-gonadotrophic activity, which reduces the concentrations of circulating sex steroids. As a result, although it is used for prostate cancer treatment, its first established indication was the control of male sexual desire and the suppression of sexually deviant behaviour (Neumann and Kalmus, 1991). Indeed, in human patients its negative impact on both androgen synthesis and action can produce effects comparable with surgical castration, with the function of androgen-dependent organs completely inhibited at high doses. This significantly reduces libido as well as sexual function. In animal models, the sexual disruption of males by cyproterone acetate has been observed to a greater extent during early development following maternal exposure. In these studies, feminisation of external gonad morphology and reduced ano-genital distance were observed in male rat foetuses to the extent that they were barely distinguishable from control females. Similar effects were also found in male dog foetuses where the absence of a prostate and a blind ended vagina were observed (Neumann, 1994).

3.1.2 Endpoints for assessing sexual disruption in fish

3.1.2.1 Secondary sexual characteristics

Secondary sexual characteristics are an important aspect of reproductive biology in many species. In males, they can be critical to an individual's reproductive fitness by determining their ability to compete with rival males and to gain female preference in mate choice. Indeed, a behavioural study in stickleback has suggested that females

prefer males with a combination of concordant traits which indicate high male quality, including strong, red throat colouration, high courtship intensity and larger body size (Künzler and Bakker, 2001). As a result, these characteristics are linked to an individual's fecundity and their mating strategy. In some species, alternative reproductive strategies exist in individuals who are less able to compete successfully in antagonistic bouts with rival males. One such strategy is female mimicry, in which "sneaky" males display secondary sexual characteristics, such as colouration, which mimic those of females. They then parasitize the efforts of other males to achieve "sneak" fertilisations by deception, a tactic which has been observed in a number of fish species (Gonçalves *et al.*, 1996; Taborsky, 1994).

For reproducing fathead minnows, males enter small, quiet waters on stream or lake margins to establish territories around spawning sites on the underside of vegetation or submerged objects. They aggressively compete for these sites and defend them from rival males by head butting behaviour, which makes the presence of the keratinous nuptial tubercles around the head and jaw advantageous (Cole and Smith, 1987; McMillan and Smith, 1974). A number of females can lay their eggs at a single male's spawning site and after fertilisation the male guards and maintains the eggs until hatch. Here the fatpad plays an important role as it is used to clean the eggs and the spawning site. It also secretes a mucus that may improve the eggs attachment to the spawning surface, as well as their overall survival through increased disease and parasite resistance (McMillan and Smith, 1974; Smith and Murphy, 1974). As a result, tubercles and fatpads are important factors in an individual male's reproductive fitness and fecundity.

Secondary sexual characteristics are modulated by endogenous hormones and in fathead minnows it is clear that androgens play an important role in their development in males. Androgens have been shown to induce these characteristics in females in laboratory exposures (Margiotta-Casaluci *et al.*, 2013b; Panter *et al.*, 2004; Ankley *et al.*, 2001) and this effect can be reversed by co-exposure to anti-androgens (Ankley *et al.*, 2004). In addition, reductions in concentrations of circulating 11-ketotesterone and testosterone have been found to coincide with reductions in tubercles in males exposed to oestrogens, which can also cause reductions in the prominence of these characteristics (Salierno and Kane, 2009; Brian *et al.*, 2007; Miles-Richardson *et al.*, 1999). Alterations to secondary sexual characteristics have also been observed in wild fathead minnows in WWTW effluent and pulp mill effluent contaminated areas (Tetreault *et al.*, 2012; Munkittrick *et al.*, 1998). Other species have also been affected, such as sand gobies captured from estuaries in the UK, where oestrogenic activity was

detected. In these fish, abnormalities in the morphology of the urogenital papilla, indicative of feminisation, occurred in over 50% of captured fish in some instances (Kirby *et al.*, 2003).

3.1.2.2 Gross indicies

These gross indices are commonly used indicators of fish health which can provide an assessment of an animal's energy allocation and stage of development. Whilst these are not as sensitive as more in depth techniques such as histology or plasma steroid and vitellogenin concentrations, they provide a good initial screening biomarker to indicate exposure effects (Lenhardt *et al.*, 2009; Pope *et al.*, 2007). Indeed, Gonadosomatic Index (GSI) provides an estimate of the gonadal development in a fish, which has been linked with reproductive status and can be disrupted by EDCs (Singh and Singh, 2008; Filby *et al.*, 2007b; Hoffmann and Oris, 2006; Ankley *et al.*, 2002; Ankley *et al.*, 2001). In comparison, Hepatosomatic Index (HSI) is of interest since the liver is the production site for vitellogenin (Sumpter and Jobling, 1995). It mainly gives an indicator of fish health, since excess energy is stored in the liver (Pope *et al.*, 2007; Plante *et al.*, 2005).

3.1.2.3 Blood plasma vitellogenin

Vitellogenin is a glycolipophosphoprotein precursor to egg yolk proteins in oocytes which provide an energy reserve to the developing embryo (Marin and Matozzo, 2004). Its synthesis in the liver is under multi-hormone control, although E2 is dominant in this process (Sumpter and Jobling, 1995). It plays an important role in gametogenesis in female fish, as it is transported in the blood to the ovary where it is rapidly sequestered by maturing oocytes. Consequently, it is responsible for extensive oocyte growth and can make up 95% of the total oocyte size in some species dependant on their reproductive strategy. For example, in fish spawning and laying buoyant eggs, which are transported in the water column, there is less vitellogenin present than those that are laid in the substrate (Tyler and Sumpter, 1996). Seasonal variation in plasma concentrations have also been observed in wild fish species in both males and females (Katsiadaki *et al.*, 2012; Hotta *et al.*, 2003). Nonetheless, due to its association with oocyte development and circulating oestradiol, vitellogenin concentrations in males are normally significantly lower or even non-detectable. However, vitellogenin can be rapidly induced to concentrations comparable with normal females following exposure to oestrogens, making it a highly sensitive biomarker of oestrogenic contamination

(Sumpter and Jobling, 1995). Indeed, vitellogenin induction has been observed in male fish exposed to steroid oestrogens as well as other oestrogenic EDCs (Brian *et al.*, 2005; Routledge *et al.*, 1998). It has also been observed in effluent-contaminated rivers, as well as in estuaries and the marine environment (Scott *et al.*, 2007; Scott *et al.*, 2006; Kavanagh *et al.*, 2004; Kirby *et al.*, 2004; Matthiessen *et al.*, 1998b; Jobling *et al.*, 1998). In addition, some anti-androgenic chemicals, such as flutamide, have also been shown to impact vitellogenin in males and females (Martyniuk *et al.*, 2009; Kang *et al.*, 2006; Panter *et al.*, 2004; Jensen *et al.*, 2004).

3.1.2.4 Intersex

Intersex is defined as the simultaneous presence of male and female gonadal tissue in a gonochoristic species (Bahamonde *et al.*, 2013). Since the discovery of its widespread occurrence in wild roach in UK rivers, intersex has been identified in 37 wild caught fish species from 17 families in 24 countries globally (reviewed by Bahamonde *et al.*, 2013). In roach, the intersex condition has been described in detail based on the gonad histopathology of 150 wild caught fish by Nolan *et a*l., which found variation in the severity of intersex amongst the sampled populations (Nolan *et al.*, 2001). A majority of fish exhibited low severity intersex, characterised by predominantly male gonad tissue with sporadically occurring primary or secondary oocytes at a varying frequency. However, more severely affected fish were found to have large proportions of the gonad made up of female oocyte tissue. Feminised reproductive ducts (ovarian cavities) were also observed in some fish alongside or independently of testicular oocytes. In females, these are characterised by two mesovarian connections between the gonad and the coelomic epithelium, creating a cavity which is used to store eggs before release during spawning (Nolan *et al.*, 2001; Lahnsteiner *et al.*, 1997). In normal males, only one connection exists between the gonad and the coleomic epithelium but malformations have been identified in which the sperm duct also develops an attachment to produce the cavity. In some severe cases, the sperm duct can be blocked or even replaced by a complete ovarian cavity (Nolan *et al.*, 2001). The presence or absence of these characteristics of intersex is dependent on the timing of exposure, with ovarian cavities only occurring after an exposure during early development (van Aerle *et al.*, 2002; Rodgers-Gray *et al.*, 2001). In contrast, although testicular oocytes can also be induced during early gonad development, they can also be produced by adults during post spawning gametogenesis (Baynes *et al.*, 2012). Indeed, there is evidence of temporal differences in intersex with a cycle of

development where intersex increases prior to spawning and redevelops post spawning (Blazer *et al.*, 2012).

Following the observation of a low prevalence of intersex in reference sites, there has been some debate as to whether there is a natural background incidence of intersex within gonochoristic fish populations (Maltret-Geraudie *et al.*, 2008; e.g. Jobling *et al.*, 1998). However, environmental influences, such as the migration of fish between pristine and polluted areas, are likely to impact such findings. In addition, a study completed at an enclosed former sand quarry in France, which received no anthropogenic discharge, did not detect intersex in the 474 roach sampled over 18 months (Geraudie *et al.*, 2010). In the Japanese medaka, histological examination of 30,000 fish also found no intersex gonads (Yamamoto, 1975). Nonetheless, intersex has been observed in negative controls from chemical exposure studies (Grim *et al.*, 2007), although this was also thought to be a result of environmental factors, such as the use of fish food containing phytoestrogens.

3.1.3 Aims and objectives

Both anti-androgenic and oestrogenic contaminants occur simultaneously in the environment, where both activities have been detected together in environmental samples, such as WWTW effluent (Johnson *et al.*, 2007b). Although these contaminants can affect common endpoints, such as intersex in fish (León *et al.*, 2007; Metcalfe *et al.*, 2001), there has been little *in vivo* study of their effects in combination. Nonetheless, an environmental modelling study of the effects of oestrogens and anti-androgenic activity on sexual disruption in fish in UK rivers, conducted by Jobling *et al.*, suggests that they could interact (Jobling *et al.*, 2009). Indeed, modelling of the associations between the predicted environmental concentrations of steroid oestrogens, nonylphenol and anti-androgenic activity in flutamide equivalents, suggested that anti-androgens had a strong additive effect on vitellogenin induction and intersex in combination with the oestrogens (Jobling *et al.*, 2009). It could be argued that a combination of bicalutamide and cyproterone acetate could result in total androgen blockade through the reduction in sex steroid synthesis and the antagonism of the receptor. This could make the fish more sensitive to sexual disruption by exogenous oestrogens.

This modelling study, along with data from mixtures toxicology studies, provided the impetus to assess the anti-androgenic pharmaceuticals in an environmentally relevant mixture scenario, informed by modelled environmental concentrations from chapter

two. Consequently, this study aimed to determine whether the two anti-androgenic pharmaceuticals, bicalutamide and cyproterone acetate, could cause sexual disruption at predicted environmental concentrations in model fish species alone, or in combination with steroid oestrogens. This was assessed in two experiments using two appropriate fish models for the endpoints of vitellogenin induction, secondary sexual characteristics and intersex incidence and severity. Since the prescriptions of the two pharmaceuticals differed considerably in the UK and Australia, UK relevant exposure scenarios were employed in these studies based on predicted concentrations from effluents and the Erewash river catchment.

3.2 Materials and methods

3.2.1 Experimental test chemicals

Bicalutamide ≥98% (CAS No. 90357-06-5), cyproterone acetate ≥98% (CAS No. 427-51-0), oestrone (E1) ≥99% (CAS No. 53-16-7), 17β-oestradiol (E2) ≥99% (CAS No. 50-28-2) and 17α-ethinylestradiol (EE2) ≥98% (CAS No. 57-63-6) were purchased from Sigma-Aldrich, Gillingham (UK). Concentrated stock solutions were prepared with *N*,*N*-dimethylformamide (DMF) ≥99.8% (CAS No. 68-12-2) purchased from Fisher Scientific, UK for tank dosing. In experiment two, stocks were also prepared in Absolute Ethanol 100 AR (CAS No. AR 64-17-5) purchased from Hayman Speciality Products, UK for petri dish dosing.

3.2.2 Experiment one: secondary sexual characteristics and vitellogenin induction

3.2.2.1 Test species: fathead minnows (*Pimephales promelas*)

Pre-spawning, male fathead minnows of approximately seven months of age were obtained from stock maintained at Brunel University, London (UK). These were maintained in 150 L tanks under flow through conditions at 25±1^{o}C with a photoperiod of 16:8 hours light:dark and a 20 minute simulated dusk/dawn period of low light. They were fed twice daily, to satiation, with flaked food (Tetramin Flake, ZM Fish Food, UK) supplemented with frozen brine shrimp (Tropical Marine Centre, Gamma irradiated) once daily.

Figure 3.1 *Male (upper) and female (lower) fathead minnows (Pimephales promelas) showing their differences in morphology. The male shows secondary sexual characteristics including nuptial tubercles (black arrows) and the fatpad (white arrow), which are absent in the female.*

Fathead minnows are a small, freshwater species belonging to the Cyprinidae family and are found broadly distributed across North America (Ankley and Villeneuve, 2006). They are gonochoristic and show clear sexual dimorphism in their secondary sexual characteristics. In males, prominent secondary sexual characteristics include nuptial tubercles, which occur mainly between the mouth and nares as well as the lower jaw, and the fatpad between the head and dorsal fin (Jensen *et al.*, 2001) (Figure 3.1). In addition, males have a much rounder head than the sharper snouted females and display black bands on the body with a black fin spot on the dorsal fin.

Having been used in a variety of testing programs, there are a number of experimental protocols available for the use of fathead minnows in ecotoxicology studies, particularly those involving endocrine disrupting chemicals (EDCs). For this study, they are an ideal species since they are larger than other commonly used fish models, such as zebrafish or Japanese medaka, so a greater blood volume can be collected for analysis of vitellogenin. Indeed, a basic assay procedure can induce a vitellogenin response in

as little as 14 days and enzyme-linked immunosorbant assay (ELISA) is available specifically for this species for accurate and robust quantification (Eidem *et al.*, 2005; Panter *et al.*, 2002). In addition, their secondary sexual characteristics are androgen-dependent and have been used in previous assessments of anti-androgens (e.g. Martinović *et al.*, 2008; Filby *et al.*, 2007b). Mechanistic studies have also used androgens to masculinise females with the induction of these secondary sexual characteristics, an effect which is blocked in combined exposure with anti-androgens (Ankley *et al.*, 2004).

3.2.2.2 Experimental design

Experiment one was carried out using an experimental system (described by Brian *et al.*, 2005) in which fathead minnows were exposed to defined mixtures of steroid oestrogens and anti-androgenic pharmaceuticals for 14 days at concentrations derived from the modelling studies. There were six treatment groups which included aquarium water and solvent controls, a steroid oestrogen treatment (E1, E2 and EE2), an anti-androgenic pharmaceutical treatment (bicalutamide and cyproterone acetate) and a combined mixture treatment, containing both anti-androgenic pharmaceuticals and steroid oestrogens. A positive control of EE2 was also employed and all treatments were replicated in duplicate with eight male fish per tank (16 per treatment). The flow through dosing system is illustrated below in Figure 3.2.

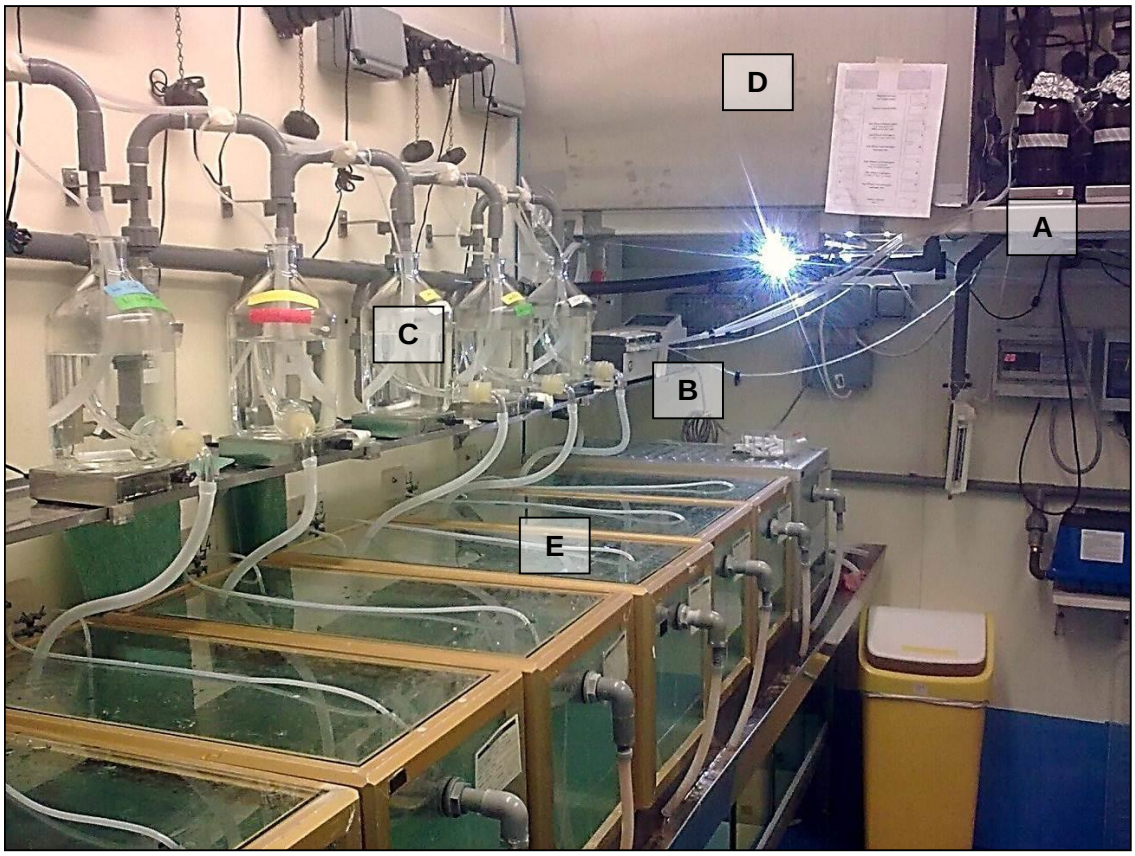


Figure 3.2 *The experimental set up for dosing exposure tanks. Solvent carrier from chemical stock bottles (A) was pumped through silicone tubing via peristaltic pump (0.01 mL/min) (B) to aspirator bottles (C), where it was diluted in aquarium water from the header tank flowing at 30 L/hour (D). This produced the desired experimental concentrations which flowed down to the exposure tanks (E) via silicone tubing.*

Chemical stocks were produced in amber bottles at 50,000× the required exposure concentration in 1 L DMF and were pumped by peristaltic pump through medical grade silicone tubing (Watson Marlow, UK) to mixing vessels at a rate of 0.01 mL/min. This was mixed with dechlorinated and filtered tap water from a header tank flowing at 30 L/hour via medical grade silicone tubing (VWR, UK), to produce the desired concentrations in the 30 L exposure tank without exceeding 20 µL/L of DMF (Hutchinson *et al.*, 2006). Each tank had its own mixing vessel, although single stock bottles fed both replicates and the negative control ran directly off the header tank. Physical and physicochemical conditions were maintained at 25±1^{o}C with dissolved oxygen exceeding 70% of air saturation and a photoperiod of 16:8 hours light:dark with a 20 minute simulated dusk/dawn period of low light. The fish were fed to satiation twice daily with flaked food (Tetramin Flake, ZM Fish Food, UK) and once daily with

frozen adult brine shrimp (Tropical Marine Centre, Gamma irradiated). Nitrite, nitrate and ammonia concentrations, as well as pH, were also monitored throughout the experiment on a weekly basis.

3.2.2.3 Experimental chemical concentrations

An environmentally relevant exposure scenario of anti-androgenic pharmaceuticals was employed, based on the concentrations predicted in chapter two for the WWTW UK1: 115 ng/L bicalutamide and 74 ng/L cyproterone acetate. These were the highest concentrations predicted from the four UK WWTWs, assuming that no removal of the pharmaceuticals would take place during wastewater treatment. Since the steroid oestrogens occur in the environment together, E1, E2 and EE2 were combined in the oestrogen treatment. This mixture of oestrogens was required to be relevant to WWTW effluents and needed to induce a submaximal response to ensure that any additional impact of anti-androgens could be detected in the mixture treatment. A concentration of 0.3 ng/L EE2 was employed based on the lowest detected concentration in WWTW effluents during a UK wide survey (Johnson *et al.*, 2007b). This was combined with 9.6 ng/L E1 and 2.1 ng/L E2, based on the ratios of the steroid oestrogens at the highest concentrations detected in the survey (3.2 ng/L EE2 : 22 ng/L E2 : 100 ng/L E1). This produced a mixture with a 17β-oestradiol equivalent concentration (EEQ) of 8.3 ng/L. To ensure that this would induce a submaximal response, the concentrations were input into the following equation for concentration addition (Thorpe *et al.*, 2003). This found that the mixture potency was below the EC50 for vitellogenin induction.

$$P_{EC50} = \frac{C_{E1}}{EC50_{E1}} + \frac{C_{E2}}{EC50_{E2}} + \frac{C_{EE2}}{EC50_{EE2}}$$

Where *P* is the proportion of the EC50 and *C* is the concentration of each steroid oestrogen in ng/L. The EC50 values were determined from the literature based on dose response studies of the effects of steroid oestrogens on vitellogenin induction in fathead minnows. These were 25 ng/L E2 and 0.9 ng/L EE2 (from Brian *et al.*, 2005) and 75 ng/L E1 (estimated from Panter *et al.*, 1998). In comparison, a t concentration of 10 ng/L EE2 was employed in the positive control.

Based on this the treatment groups and doses were as follows:

- Control
- Solvent Control

- Anti-Androgens (115 ng/L bicalutamide; 74 ng/L cyproterone acetate)
- Steroid Oestrogens (0.3 ng/L EE2; 9.6 ng/L E1; 2.1 ng/L E2)
- Mixture (115 ng/L bicalutamide; 74 ng/L cyproterone acetate; 0.3 ng/L EE2; 9.6 ng/L E1; 2.1 ng/L E2)
- Positive Control: 10 ng/L EE2

3.2.2.4 Fish sampling

After 14 days of chemical exposure, the fish were sacrificed by a lethal dose of MS222 (500 mg/L) buffered to pH 7.4 according to UK Home Office Regulations. Blood was collected by caudal fin amputation with heparinised microhaematocrit tubes. The samples were then centrifuged at 7000 g for 5 minutes at 4^{o}C to collect plasma, which was stored at -80^{o}C. Fork length and wet weight were recorded to determine condition factor. Livers and gonads were excised following ventral incision and were weighed to assess liver and gonad development. Formulas for the condition factor, GSI and HSI are detailed below:

$$Condition\ Factor\ = \frac{body\ weight\ (g)}{body\ length\ (cm)^3} \times 100$$

$$HSI\ = \frac{liver\ weight}{body\ weight} \times 100$$

$$GSI\ = \frac{gonad\ weight}{body\ weight} \times 100$$

3.2.2.5 Assessing secondary sexual characteristics

In this experiment, the number of tubercles on each fish was counted and their prominence was scored based on criteria displayed in **Table 3.1** (Smith, 1978). In addition, the fatpad was removed from each fish and its weight recorded to calculate fatpad index based on the equation below:

$$Fatpad\ Index\ = \frac{fatpad\ weight}{body\ weight} \times 100$$

Table 3.1 *The scoring system for tubercle prominence (based on Smith, 1978).*

Score	Description of nuptial tubercles
0	no visible sign of tubercles
1	tubercles visible as white disks, not protruding above body surface
2	tubercles project above body surface
3	tubercles prominent but not sharp
4	tubercles prominent and sharp
5	tubercles have started to run together and not all individual tubercles can be distinguished

3.2.2.6 Vitellogenin analysis

In this experiment, 10 µL plasma samples from individual fish were analysed by ELISA, specific to fathead minnows vitellogenin (Biosense Laboratories AS, Norway) following the instructions of the manufacturer. The test kit employed is a sandwich ELISA, in which vitellogenin in plasma samples is bound between a capture antibody within the wells of 96 well microplates and an enzyme linked detection antibody, which is added later in the process. Binding of the detection antibody activates the enzyme to produce a colour change in the substrate within the wells, which is directly proportional to the amount of vitellogenin present in the sample (Biosense Laboratories, 2005). In this experiment, the colour absorbance was measured at 450 nm with a Spectramax Plate Reader (Molecular Devices, USA) and used to calculate the concentration of vitellogenin. Due to the high concentrations anticipated in some samples, particularly in oestrogen exposed groups, the plasma samples were diluted with buffer solution to keep the concentrations within the working range of the assay (Table 3.2).

Table 3.2 *The dilutions of plasma from fish in each treatment group used in the ELISA.*

Treatment	Dilutions of 10 µL plasma sample
Control	1:50
Solvent Control	1:50
Oestrogens	1:5000, 1:500,000
Anti-Androgens	1:50, 1:5000
Mixture	1:50, 1:5000, 1:500,000
Positive Control	1:5,000,000, 1:50,000,000

3.2.2.7 Statistical analysis

To determine variation and differences between treatment groups, the data were tested for normality and equal variance. Parametric data then underwent one way analysis of variance (ANOVA) followed by post hoc all pairwise multiple comparison procedures (Holm-Sidak Method). Non-parametric data was assessed by Kruskall-Wallis one way ANOVA on ranks followed by post hoc all pairwise multiple comparison procedures (Dunn's Method). Vitellogenin data were Log transformed prior to assessment and statistical significance was accepted at $P \leq 0.05$ for all tested endpoints. Treatment groups were all compared with the solvent control. These analyses were performed in SigmaStat 3.5 (Systat Software, Chicago IL).

3.2.3 Experiment two: intersex induction in Japanese medaka

3.2.3.1 Test species: Japanese medaka (*Oryzias latipes*)

Japanese medaka were obtained from a stock maintained at Brunel University, London (UK), which originated from the National Institute for Environmental Studies, Japan. Broodstock were kept as triplets of one male to two females to maximise egg production under flow through conditions in 8 L tanks at 27±1^{o}C, with a photoperiod of 16:8 hours light:dark and a 20 minute simulated dusk/dawn period of low light. Their diet consisted of pellet food (ZM Granular, ZM Fish Food, UK) supplemented with frozen adult brine shrimp once daily (Tropical Marine Centre, Gamma irradiated). Fish in all tanks were fed to satiation.

Figure 3.3 *Adult Japanese medaka (Oryzias latipes) maintained in a stock tank.*

Japanese medaka are a small, freshwater, gonochoristic fish species belonging to the Adrianchythyidae family (Figure 3.3). They originate from South East Asia and have also become a well-established model species for ecotoxicology testing programs, with a number of advantages supporting their use in early life studies and assessments of intersex induction (Urushitani *et al.*, 2007). Critically, they have a short life cycle and can reach maturity within 100 days, with gonadal sexual differentiation observed at around 12 days post hatch in comparison to 80-90 days for fathead minnows (Hirai *et al.*, 2006; Ankley and Johnson, 2004). In addition, unlike zebrafish, they do not go through a pseudo-hermaphroditic stage during early development (Maack and Segner, 2004). The adults also show clear sexual dimorphism in their secondary sexual characteristics, which include differences in the shape of the anal fin, making them easy to pair in breeding scenarios. Some strains also show sex dependent colouration, such as the FLF-II strain, where males are orange and females are white. In addition, females are oviparous and can spawn daily with the right photoperiod and feeding regime (Arcand-Hoy and Benson, 1998), producing 10-30 eggs per day (Yamamoto,

1975) which ensures a relatively constant supply of embryos for early life studies. The embryos themselves are transparent so embryonic development can be observed throughout the process and abnormalities can be easily identified.

Medaka are also sexually dimorphic at a genetic level with XX females and XY males as well as a sex specific DMY gene marker, which is present only in males. Indeed, medaka are one of the few fish species to have a well-established sex determining gene. In comparison, a sex determining gene has only been recently discovered in the fathead minnows (Olmstead *et al.*, 2011). The use of this gene in medaka can overcome the diagnostic issue of determining whether intersex induction by complex mixtures can occur due to masculinisation of females or feminisation of males, which has caused complications in the interpretation of data collected from wild roach and those used in effluent exposures (Lange *et al.*, 2009; Jobling and Tyler, 2003). Indeed, intersex can be induced in medaka by a variety of EDCs, including the steroid oestrogens, bisphenol A (BPA), pesticides and pharmaceuticals (Kiparissis *et al.*, 2003; Metcalfe *et al.*, 2001).

3.2.3.2 Experimental design

Japanese medaka were exposed from an embryonic to early adult stage over 98 days. As in experiment one, fish were treated with the anti-androgenic pharmaceuticals, the steroid oestrogens and a combined activity mixture. However, in this experiment, the treatment regime was repeated at two different doses termed “high” and “low.” These were run alongside solvent and aquarium water controls with a positive control of 50 ng/L E2 to give a total of nine treatments. All treatment sets were replicated in duplicate with 25 fish per tank (50 per treatment). Embryos were collected and exposure was initiated at less than six hours post fertilisation. The embryos were maintained under static renewal in covered glass petri dishes with 15 mL of Modified FETAX (Frog Embryo Teratogenesis Assay-Xenopus) Solution (MFS) (Woods and Kumar, 2011) in an incubator maintained at 25±1^{o}C with 16:8 hours light:dark ratio.

Table 3.3 *The constituents of Modified FETAX Solution (MFS) required for producing 2.5 L of solution, purchased from Sigma-Aldrich, UK* (from Woods and Kumar, 2011).

Compound	CAS #	Weight (mg) to add to 2.5 L double distilled water
Magnesium Sulphate	7487-88-9	187.5
Sodium Bicarbonate	144-55-8	240
Calcium Chloride	10043-52-4	37.5
Sodium Chloride	7647-14-5	1000
Potassium Chloride	7447-40-7	75
Calcium Sulphate dihydrate	10101-41-4	150

MFS water was produced based on Table 3.3. Calcium sulphate dehydrate was added to 2.5 L of double distilled water in a 2.5 L Winchester bottle and was dissolved overnight using a magnetic stirrer. The rest of the salts were mixed in the following day and left for an additional 24 hours to dissolve fully. The solution was kept aerated with an air stone to ensure that there was sufficient dissolved oxygen available.

Over the first three days of exposure in petri dishes, unfertilised or non-viable embryos were replaced with newly fertilised embryos to maintain a number of 25 embryos per dish/tank in the early phases of the study. Each petri dish was spiked with 10 µL of a concentrated stock solution in absolute ethanol, which was allowed to evaporate before 15 mL of MFS was added. Although this could be described as a "solvent free" technique, the solvent control was still spiked with ethanol only. Embryos were transferred by pipette to new petri dishes, with new stocks replenished daily to provide 24 hour renewal of the exposure mixtures. As the embryos hatched, larvae were transferred to 30 L exposure tanks run with the same water and chemical stock flow regimens as experiment one. They were maintained in mesh baskets for up to 14 days before being released into the aquarium itself. Temperature was maintained at 26±1^{o}C throughout the majority of the study. However, a fault with the heating element in the header tank caused a drop in temperature of up to 2.7^{o}C in the majority of the tanks, which was quickly fixed, causing the temperature to return to normal within 24 hours. Another temperature decrease was observed later in the experiment in the control tank (A), high anti-androgen (B) and low oestrogen (B). This was caused by a reduction in the inflow of water from header tank to the treatment tanks and was quickly rectified, with temperature returning to normal in the next daily measurement. Control tank (A) experienced much greater variation in temperature range (5.7^{o}C) than the other tanks. Dissolved oxygen exceeded 70% saturation throughout the study in all tanks with the exception of the first high anti-androgen replicate, where 68% saturation was

experienced at one sampling point. In addition, a photoperiod of 16:8 hours light:dark with a 20 minute simulated dusk/dawn period of low light was applied and fish were fed twice daily to satiation with pellet food. This was supplemented with larval artemia and eventually frozen brine shrimp. Nitrite, nitrate and ammonia concentrations and pH were also monitored throughout the experiment.

3.2.3.3 Experimental chemical concentrations

As in experiment one, treatment concentrations of anti-androgenic pharmaceuticals were based on concentrations predicted from the UK WWTW effluents in chapter two. In this experiment, the predicted steroid oestrogen concentrations were also employed to simulate the predicted environmental scenarios. For the high doses, the treatment concentrations originated from UK1: 115 ng/L bicaltuamide, 74 ng/L cyproterone acetate, 29 ng/L E1, 4 ng/L E2 and 0.4 ng/L EE2 (18 ng/L EEQ). This was intended to produce an exposure scenario relevant to high WWTW effluent concentrations. However, since anti-androgenic pharmaceuticals were expected to occur at lower concentrations in UK rivers, based on data in chapter two, a second set of concentrations were used in the low dose treatments. These were based on the lowest predicted effluent concentrations, which occurred at UK3: 53 ng/L bicalutamide, 34 ng/L cyproterone acetate, 13 ng/L E1, 2 ng/L E2, 0.2 ng/L EE2 (8 ng/L EEQ). These are very similar to the maximal concentrations predicted to occur in the River Erewash, which were 50 ng/L bicalutamide, 32 ng/L cyproterone acetate, 12 ng/L E1, 1.7 ng/L E2 and 0.17 ng/L EE2 (7.4 ng/L EEQ). In addition, the steroid oestrogen concentrations were expected to induce a background incidence of intersex of around 10% and 20% respectively for the low and high concentrations, based on a comparison between their combined EEQ and a dose response of E2 in medaka for the induction of intersex (Metcalfe *et al.*, 2001). Finally, the positive control was dosed at 50 ng/L E2 based on concentrations required for complete sex reversal of males (Kinoshita *et al.*, 2009).

To reiterate, the following treatments and doses were employed

- Control
- Solvent Control
- Low Anti-Androgens (53 ng/L bicalutamide; 34 ng/L cyproterone acetate)
- Low Steroid Oestrogens (13 ng/L E1; 2 ng/L E2; 0.2 ng/L EE2)
- Low Mixture (53 ng/L bicalutamide; 34 ng/L cyproterone acetate; 13 ng/L E1; 2 ng/L E2; 0.2 ng/L EE2)

- High Anti-Androgens (115 ng/L bicaltuamide; 74 ng/L cyproterone acetate)
- High Steroid Oestrogens (29 ng/L E1; 4 ng/L E2; 0.4 ng/L EE2)
- High Mixture (115 ng/L bicalutamide; 74 ng/L cyproterone acetate; 29 ng/L E1; 4 ng/L E2;0.4 ng/L EE2)
- Positive Control: 50 ng/L E2

3.2.3.4 Fish sampling

After 98 days exposure, medaka were sacrificed by a lethal dose of neutral buffered MS222 (500 mg/L). However, fish in the positive control treatment were sampled at 90 days, due to sporadically occuring mortality throughout the exposure. The heads and tails were removed and the bodies were fixed in bouin's solution (Sigma-Aldrich, UK) and stored in 70% IMS. Blood samples were also taken by caudal fin amputation using heparinised haematocrit tubes but were insufficient in volume to test for vitellogenin induction.

3.2.3.5 Histological processing

The preserved body cavity was opened from the right side to reveal the gonad (Figure 3.4) before being processed in an automatic tissue processor (Leica TP1020, Leica Inc.) based on Table 3.4 and fixed in wax blocks. The blocks were then serial sectioned longitudinally from the start of the gonad, with 5 µm sections transferred to microscope slides at every 150 µm. For smaller fish, the entire body was sectioned and for females with large ovaries, the gonads were excised and split longitudinally into sections before being set in wax and serial sectioned in the same fashion as the males. The slides were allowed to dry for 48 hours before being stained with haemotoxylin and eosin (H&E) using an automatic modular linear batch stainer (Stainmate, Raymond A Lamb) according to Table 3.5. They were then coverslipped using histomount.

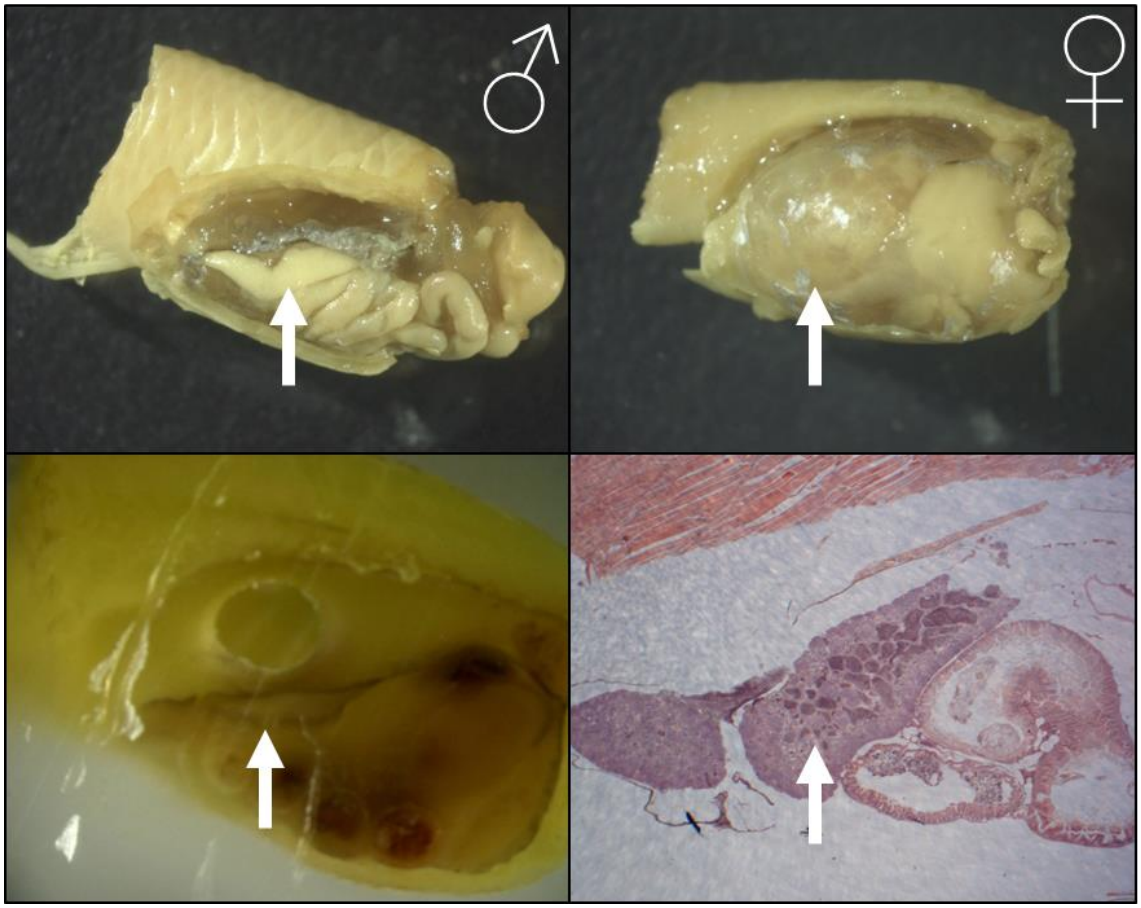


Figure 3.4 *The stages of the histological process showing a preserved male (above-left) and a female (above-right) with the body cavity opened. These were processed and set in wax (male below-left) and serial sectioned for microscopy (male below-right). The gonad is indicated with white arrows in each image.*

Table 3.4 *The tissue processing protocol for the histology of Japanese medaka using the Leica TP1020.*

Cycle Number	Treatment	Hours
1	70% IMS	3
2	90% IMS	2.5
3	95% IMS	1.5
4	100% IMS	1.5
5	100% IMS	1.5
6	100% IMS	1.5
7	100% IMS	1.5
8	HISTOCLEAR	1.5
9	HISTOCLEAR	1.5
10	HISTOCLEAR	1.5
11	WAX	1.25
12	WAX	1.25

Table 3.5 *The staining protocol for histology of Japanese medaka using the Stainmate.*

Step	Chemical	In bath time (secs)	In bath agitation	Out bath time (secs)	Out bath agitation
0	Histoclear	900	N	8	Y
1	100% IMS	120	N	8	Y
2	90% IMS	120	N	8	Y
3	70% IMS	120	N	8	Y
4	Flowing tap water	120	N	8	Y
5	Haematoxylin	600	N	8	Y
6	Flowing tap water	600	N	8	Y
7	Acid alcohol[1]	20	N	8	Y
8	Flowing tap water	20	Y	8	Y
9	Saturated. Li_2CO_3	20	N	8	Y
10	Flowing tap water	20	Y	8	Y
11	Eosin	40	N	8	Y
12	Flowing tap water	300	N	8	Y
13	70% IMS	120	N	8	Y
14	90% IMS	120	N	8	Y
15	100% IMS	300	N	8	Y
16	Histoclear	300	N	0	N
17	Histoclear	0	N	0	N

[1]Acid alcohol was produced by mixing 1% hydrochloric acid with 70% IMS in purite water.

3.2.3.6 Intersex analysis

Intersex incidence was quantified based on the presence or absence of oocytes and ovarian cavities through light microscopy, using an Olympus BX51 compound light microscope. Intersex severity for each gonad section was then scored with an intersex index of 0-7 based on the abundance of oocytes within the gonad tissue (Table 3.6) (Jobling *et al.*, 2006). All slides were blinded prior to microscopic analysis.

Table 3.6 *The intersex index scoring system used to quantify severity in the gonad sections from sampled fish* (from Jobling *et al.*, 2006)**.**

Score	Description of the observed severity of intersex
0	Normal male testis
1	Multifocal ovotestis with 1-5 oocytes (usually singly) scattered amongst the testicular tissue
2	Multifocal ovotestis, 6-20 oocytes often in small clusters scattered amongst the testicular tissue
3	Multifocal ovotestis, 21-50 oocytes in clusters
4	>50<100 oocytes. Section is usually multifocal and has the appearance of a mosaic of testicular and ovarian tissue
5	>100 oocytes, usually multifocal but could also be focal with clearly identifiable zones of ovarian and testicular tissue separated from the testicular tissue
6	>50% of the gonadal tissue on the section is ovarian and is clearly separated from the testicular tissue by epithelial cells and phagocytic tissues
7	100% of gonadal tissue on the section is ovarian

3.2.3.7 Statistical Analysis

Binary responses (intersex incidence) were analysed using generalized linear modelling approaches, assuming that the numbers of intersex fish were binomial-distributed. Mean effects were estimated by Maximum Likelihood, and treatment-related differences to the controls were analysed by Williams contrast test (Hothorn, 2004). Ordinal responses (intersex severity) were assumed to follow a common slopes model with a cumulative logit link function, and correlations from repeated

measurements were estimated according to the principles of Generalized Estimating Equations (Agresti, 1984). Odds ratio estimates were used to express relative differences between treatment means. For the log odds scale, the cumulative logit model is sometimes referred to as the proportional odds model. Comparisons were drawn between treatment groups and the solvent controls. Analysis was performed in SAS 9.3 (SAS Institute, Cary NC).

3.2.4 Chemical Analysis

3.2.4.1 Sample collection

In experiment one, 500 mL aquarium water samples were collected on days 1 and 14 and were refrigerated in amber winchester bottles below 6^{o}C prior to extraction. These bottles had undergone silylation prior to use with 5% dimethyldichlorosilane in toluene (Sylon CT from Sigma-Aldrich, UK), to minimise chemical loss by adsorption onto the glass (Armarego and Chai, 2009). The protocol for this process can be found in appendix three. In experiment two, the sample volume was reduced to 100 mL. Samples were collected on day 20, when all fish had been transferred from petri dishes to tanks, as well as day 34, 62 and 90. These were held in silylated glass bottles and were extracted on the same day. A degradation test was also run to determine what concentrations were likely to occur in the petri dishes over a 24 hour period prior to their renewal. Here, two replicate samples containing 100 mL of aquarium water were spiked with the high concentrations of anti-androgens and oestrogens and were left in the incubator for 24 hours prior to extraction. All samples from both experiments were spiked with 5 ml/L of HPLC grade methanol (Fisher Scientific, UK) for preservation (Desbrow *et al.*, 1998) as well as 100 ng/L of the deuterated internal standards d_7 propranolol and d_{10} carbamazepine prior to extraction. Ideally, deuterated standards for the chemicals of interest would have been used because they act in a very similar fashion during the sampling and analytical methodology (Wieling, 2002). However, since these were not available for purchase for bicalutamide and cyproterone acetate, d_7 propranolol and d_{10} carbamazepine were used. These provided sufficient alternatives since they are both pharmaceutical chemicals with known and compatible analytical methods of detection.

3.2.4.2 Solid phase extraction

In this study, solid phase extraction (SPE) was used to concentrate the experimental samples to improve their detection during liquid chromatography/tandem mass spectrometry (LC-MS/MS). Experimental samples underwent reverse phase SPE on C18 cartridges (Sep-Pak C18, 360 mg sorbent, 55-105 µm particle size; Waters, UK), which can capture compounds with a broad range of polarities. A blank of double distilled water and three spiked aquarium water samples were extracted alongside the experimental samples. The spikes contained anti-androgens and oestrogens at their low concentrations (low spike), high concentrations (high spike) and E2 and EE2 at their positive control concentrations dependent on the experiment. C18 cartridges were attached to an extraction manifold and conditioned with 5 mL of HPLC grade methanol followed by 5 mL of double distilled water in sequence. Each sample was then loaded onto the cartridge under vacuum before being thoroughly dried under air. The samples were then eluted into 7 mL glass collection tubes with 5 mL of methanol. A nitrogen stream was then used to further concentrate the samples, which were blown down to 200 µL before being split into two 100 µL aliquots, stored in inserts in 1 mL autosampler vials for anti-androgen and steroid oestrogen testing respectively. Deuterated steroid oestrogen internal standards were also added to one set of aliquots at this point (2,4,16,16-d_4-oestrone, 2,4,16,16-d_4-17β-oestradiol and 2,4,16,16-d_4-17α-ethinylestradiol), at 100 ng/100 µL and samples were returned to a 100 µL volume by nitrogen blow down. This method was produced following extraction efficiency tests employing different three different pH's (4, 7 and 11), two different SPE cartridges (C18 and Oasis HLB, 500 mg sorbent, 50 µm particle size; Waters, UK) and three different elution solvents: methanol, acetonitrile and a 50/50 ethyl acetate/acetone mix. This found the use of C18 cartridges at a neutral pH with a methanol elution to be the best compromise in maintaining sufficient extraction efficiency for both anti-androgenic pharmaceuticals.

The eluted samples from both experiments were accumulated prior to analysis. At this point it became evident that fine particulate matter was present in the eluted samples after the dry down procedure. These are referred to as fines, which are fractured particles from the silica sorbent in the C18 cartridge. Their occurrence is documented in the literature when using silica based cartridges and the levels vary between batches. This could be avoided in future study by using cartridges with an alternative solid phase, such as polymerics, or by using C18 disks. During the LC-MS/MS procedure the high concentrations of fines in the samples caused blockage of the instrument on a number of occasions. As a result, the decision was made to separate

the methanol eluent from the fines by centrifugation. Here, the samples were glass pipetted to centrifuge tubes and spun at 7,000× for three minutes to separate the eluent from the fines. The methanol eluent was then transferred back to the autosampler vials for analysis by LC-MS/MS.

3.2.4.3 Liquid chromatography/tandem mass spectrometry

As part of this study, identification and quantification methods for the anti-androgenic pharmaceuticals, bicalutamide and cyproterone acetate, were developed using LC-MS/MS. Reference standard chemicals (1 µg/mL in methanol) were directly infused at 5 µL/min into the LC-MS/MS to optimise conditions for analysis before experimental samples were run. The declustering potential of the chemicals was also optimised based on Table 3.7. In contrast, the steroid oestrogens were analysed based on a previously documented methodology (Environment Agency, 2008b).

- **Steroid oestrogens**

The method for analysing steroid oestrogens was taken from the Standing Committee of Analysts book on the detection of steroid oestrogens in water samples by mass spectrometry (Environment Agency, 2008b). One 100 µL aliquot of sample extract, containing deuterated internal standards, was used to assess these chemicals, of which 30 µL was injected. The high performance liquid chromatography (HPLC) system (Aglient 1100 series) was maintained at 40^{o}C with a C18 based column (Aglient ZORBAX Eclipse XDB-C18, 150mm x 4.6 x 5µm. Part No. 993967-902). The mobile phase employed a gradient flow at 500 µL/minute using two eluents: (A) acetonitrile (Fisher Scientific, UK) and (B) 0.1% ammonium hydroxide in milliQ water. Separation started with 15% eluent A for three minutes, increasing to 40% for seven minutes, 75% for two minutes and 90% for four minutes before returning to 15%. The total sample acquisition time was 16 minutes.

Detection and quantification was carried out using an Applied Biosystems API5000 triple quadrupole mass spectrometer using negative ion electrospray in multiple reaction monitoring (MRM) mode. The use of electrospray ionisation here was particularly advantageous since it is considered to be a “soft” ionisation technique where little fragmentation occurs; making it easier to identify chemicals with common structural features (Pitt, 2009). The optimal settings were a source temperature of 550^{o}C and ion spray voltage at -4,500 V, whilst curtain gas and ion source gas one and two were set at 30, 50 and 60 psi respectively. The retention times and mass

transitions which were used to identify the chemicals are shown in Table 3.7. Since the internal standards were only added to experimental samples post extraction, quantitation was completed with a six point calibration set extracted with internal standards from 100 mL aquarium water using the extraction technique described. These contained concentrations equivalent to 0, 1, 2, 5, 10 and 50 ng/L, through which loss during extraction could be normalised in the experimental samples. Linearity was assessed by regression analysis, which found the following ions to be the most accurate to determine concentrations: E1 269.2/143.0 (R=0.9993); E2 271.1/143.1 (R=0.9990) and EE2 295.2/143 (R=0.9993). The limit of detection (LOD) of this analytical method is reported as 0.05 ng/L (Williams *et al.*, 2012).

- **Bicalutamide**

The HPLC system was maintained at 30^{o}C using the same Agilent C18 based column used for the steroid oestrogens. The second 100 µL aliquot of sample extract was used to analyse the anti-androgenic pharmaceuticals, of which a 10 µL volume was injected. The mobile phase employed a gradient flow at 1000 µL/minute using two eluents: (A) acetonitrile (Fisher Scientific, UK) and (B) 0.1% ammonium hydroxide in milliQ water. Separation started with 15% eluent A for six minutes, increasing to 95% for another six minutes before returning to 15% for the remainder of the 15 minute samples acquisition time.

Detection and quantification was carried out using an Applied Biosystems API5000 triple quadrupole mass spectrometer using negative ion electrospray in MRM mode. The optimal settings were a source temperature of 550^{o}C and ion spray voltage at -4500 V, whilst curtain gas and ion source gas one and two were set at 25, 50 and 60 psi respectively. Bicalutamide was identified by comparing the retention time and ion abundance ratios of the quantitation and confirmation mass transitions to those of reference standard chemicals (Table 3.7 and Figure 3.5). The concentrations were then calculated from a five point calibration curve using internal standard quantitation in Analyst 1.5.1 software (Thermo-Electron Corporation). The calibration curve was produced from extracts of a set of 100 mL aquarium water samples containing 0, 20, 50, 100, 200 ng/L of bicalutamide and cyproterone acetate alongside internal standards as previously described. In this case, d_7 propranolol was used as the internal standard since it was more accurately analysed in negative ion mode. Linearity was assessed by regression analysis, which found the 429.2/185.0 ion to be the most accurate determinate of concentration (R=0.9998). A LOD equivalent to 5 ng/L was determined from a solvent calibration which produced a signal to noise (S/N) ratio of 429.

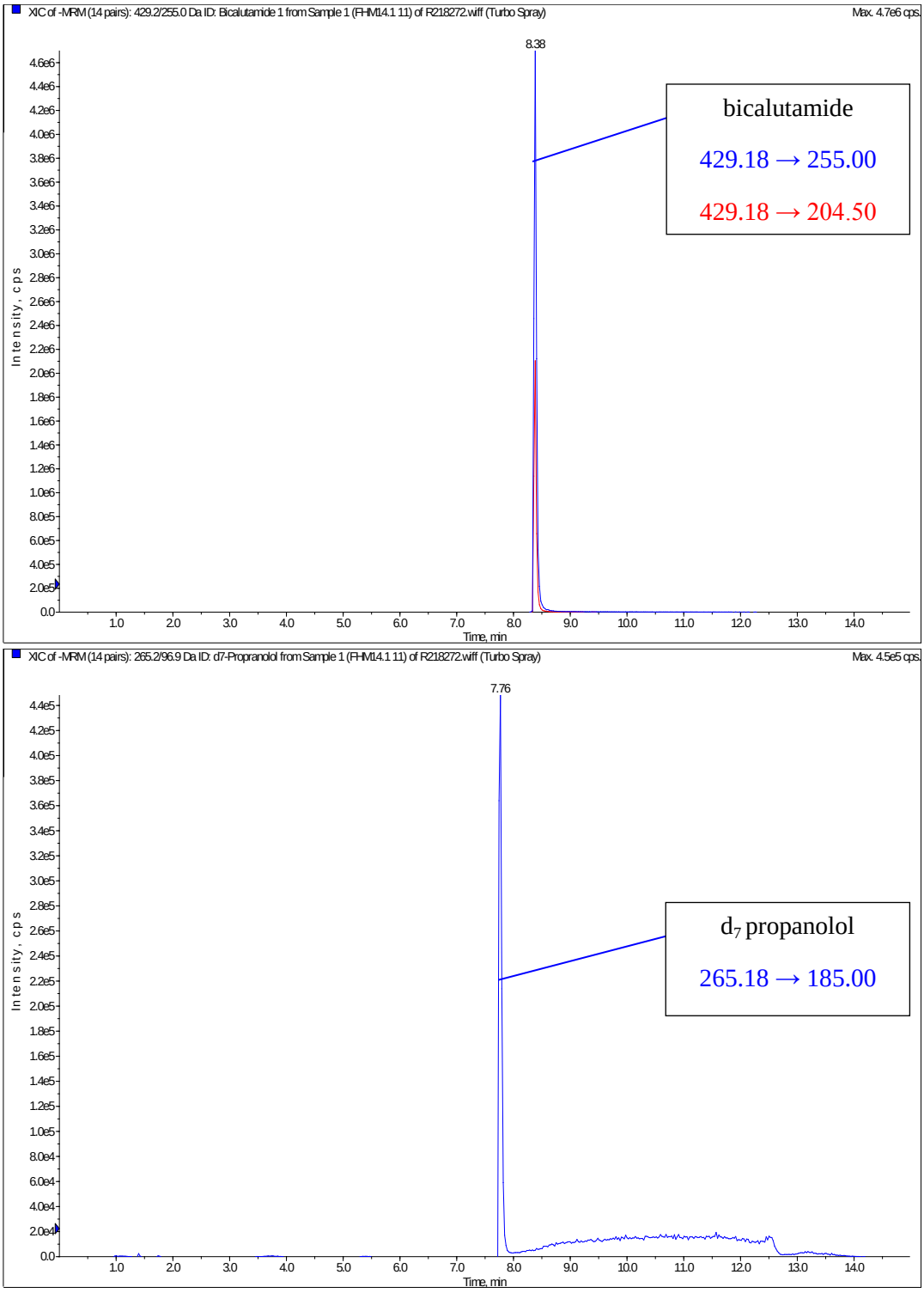


Figure 3.5 *Chromatograms of bicalutamide and the internal standard d_7 propranolol in an aquarium water sample from the mixture treatment in experiment one. This was detected by LC-MS/MS run in negative ion mode. The mass transitions are also displayed.*

- **Cyproterone acetate**

Extracts tested for bicalutamide analysis were retested for cyproterone acetate. The HPLC system was maintained at 30^{o}C using the same Agilent C18 based column. A 20 µL volume of sample was injected and the mobile phase employed a gradient flow at 1,000 µL/minute using two eluents: (A) acetonitrile (Fisher Scientific, UK) and (B) milliQ water. Separation started with 15% eluent A for six minutes, increasing to 95% for another six minutes before returning to 15% for the remainder of the 15 minute sample acquisition time.

Detection and quantification was carried out on the API5000 using positive ion electrospray in MRM mode. The optimal settings were a source temperature of 600^{o}C and ion spray voltage at 4,500 V, whilst curtain gas and ion source gas one and two were set at 25, 70 and 60 psi respectively. Cyproterone acetate was identified by comparing the retention time and ion abundance ratios of the quantitation and confirmation mass transitions to those of reference standard chemicals (Table 3.7 and Figure 3.6). The concentrations were then calculated from the five point extraction calibration curve using internal standard quantitation with d_{10} carbamazepine, which is more accurately analysed in positive ion mode. Regression analysis assessing linearity found the 417.3/43.2 ion to be the most accurate determinate of the concentration (R=0.9960). An LOD of 10 ng/L was determined from a solvent calibration set, which had a signal to noise ratio of 11.9.

Unfortunately due to a computer error, the results from the anti-androgen analysis were lost. This coincided with the shutdown of Severn Trent Services, Reading where this work was being completed, which meant access to the LC-MS/MS to reanalyse the samples was lost. Extract samples were stored at -20^{o}C until they could be re-run over 12 months later using the same methods and the same API5000 LC-MS/MS, which had been acquired by Brunel University in the meantime. However, it is possible that degradation of the samples occurred during this time as well as adsorption to the glassware. This, along with the high concentration of fines, could have created additional sources of analytical error.

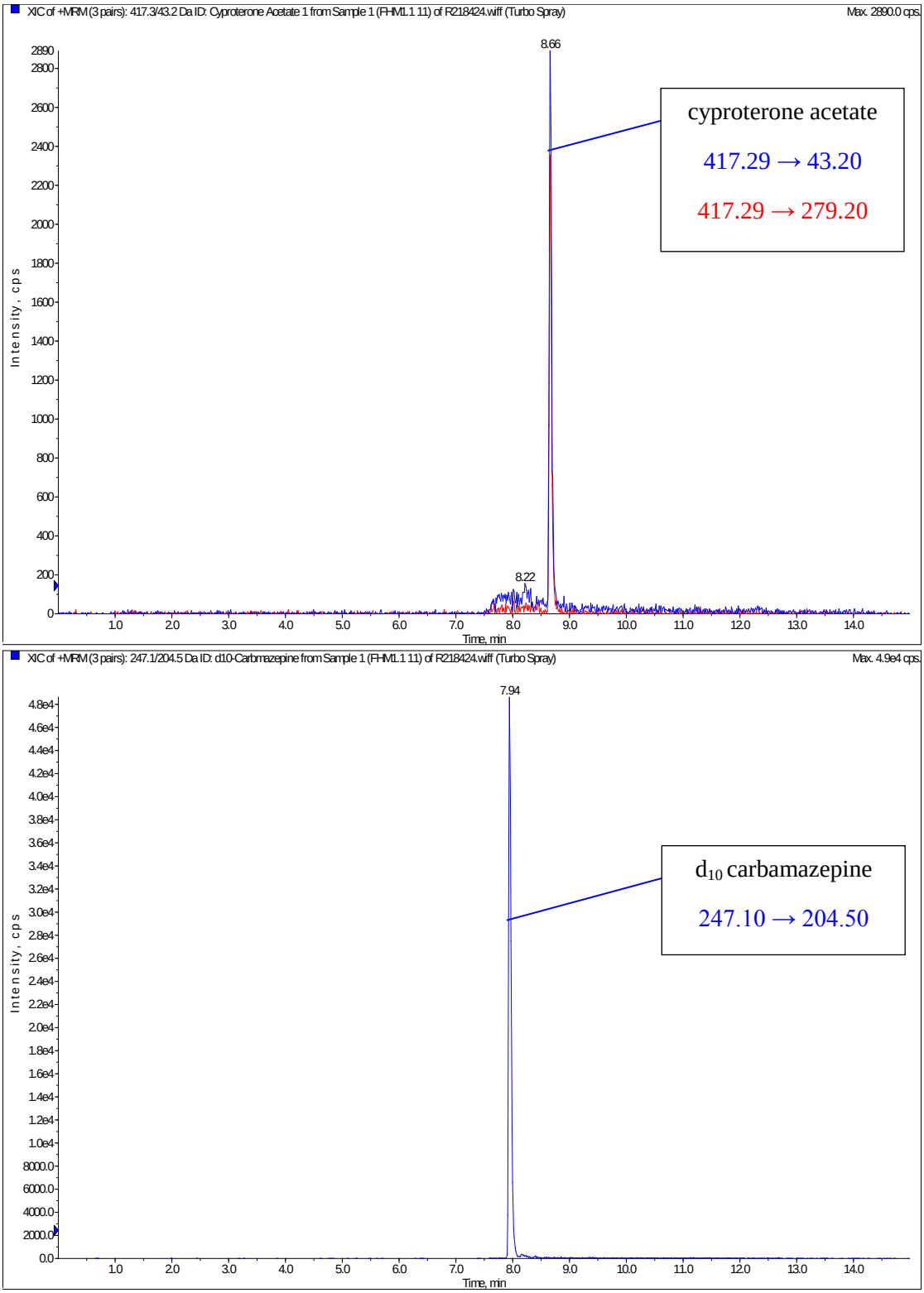


Figure 3.6 *Chromatograms showing the presence of cyproterone acetate and the internal standard d_{10} carbamazepine in an aquarium water sample from the mixture treatment from experiment two. This was detected by LC-MS/MS run in positive ion mode. The mass transitions are also displayed.*

Table 3.7 *Optimised instrumental conditions for LC-MS/MS analysis of anti-androgenic pharmaceuticals and steroid oestrogens. Internal standards are italicised.*

Compound	Ionisation Mode	Retention time (mins)	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	EP (V)	CE (V)	CXP (V)
bicalutamide	Negative	8.38	429.18	255	-115	-10	-20	-9
			429.18	185	-115	-10	-48	-31
d_7 propanolol	Negative	7.76	265.18	96.9	-125	-10	-36	-15
cyproterone acetate	Positive	8.66	417.29	43.2	96	10	117	10
			417.29	279.2	96	10	51	50
d_{10} carbamazepine	Positive	7.94	247.1	204.5	61	10	49	54
oestrone	Negative	8.5	269.16	145	-210	-10	-52	-24
			269.16	143	-210	-10	-68	-24
d_4 oestrone	Negative	8.49	273.1	147.1	-210	-10	-68	-24
17β-oestradiol	Negative	7.64	271.14	145.1	-195	-10	-54	-21
			271.14	143.1	-195	-10	-54	-19
d_4 17β-oestradiol	Negative	7.6	275.15	147.1	-195	-10	-54	-19
17α-ethinylestradiol	Negative	8.15	295.15	145.1	-190	-10	-54	-19
			295.15	143	-190	-10	-54	-23
d_4 17α-ethinylestradiol	Negative	8.12	299.15	147.1	-190	-10	-54	-23

Q1 mass (parent ion m/z); Q3 mass (daughter ion m/z); DP (declustering potential); EP (entrance potential); CE (collision energy); CXP (cell exit potential).

3.2.5 UK catchment modelling of anti-androgenic pharmaceuticals

In order to put the exposure concentrations into context and to produce a more extensive picture of environmental contamination by the anti-androgenic pharmaceuticals in the UK, predictive modelling techniques used in chapter two were expanded. The Low Flows 2000 Water Quality eXtension model (LF2000-WQX) was used to predict their concentrations in 14 major river catchments in England and Wales, which accounted for approximately 54% of land area and 69% of the population served by WWTWs. The per capita loads which were used in the Erewash model were used again for this catchment modelling with no removal incorporated at the WWTWs. Again, with no data available on the in-stream degradation of the drugs, the model produced a worst case scenario for their environmental concentrations, only taking into account the effects of dilution of the effluent in downstream river stretches. The predicted concentrations were compared to experimental concentrations used in the two experiments to further assess their environmental relevance.

3.3 Results

3.3.1 Experiment one

3.3.1.1 Chemical analysis

Aquarium water samples were collected from all treatment tanks on days 1 and 14 of the experiment. Concentrations and the % of the nominal concentrations achieved are displayed in Table 3.8. All chemicals were successfully detected in the appropriate samples. Cyproterone acetate was successfully detected in the anti-androgen and mixture treatment tanks with peaks exceeding three times the signal to noise ratio. However, it could not be accurately quantified due to the unreliable, variable detection and quantification of the internal standard (d_{10} carbamazepine). Concentrations of cyproterone acetate were instead predicted based on its expected ratio to bicalutamide, with which it had been combined in the stock solutions. This estimated concentrations to be between 47-107 ng/L (63-144% of nominal). For the other chemicals, concentrations in the tanks varied above and below the nominal, ranging from 3.9-12.8 ng/L (41-133% of nominal) for E1, 0.7-2.7 ng/L (32-133%) for E2, 0.2-0.3 ng/L (33-113%) for EE2 and 73-166 ng/L (63-144%) for bicalutamide. In the positive control, concentrations of EE2 ranged from 7.0-11.6 ng/L (70-116% of nominal). Replicate tanks showed reasonably good agreement in the concentrations of bicalutamide and the steroid oestrogens, as did the single and combined mixture treatments at the start of the experiment. Concentrations decreased on day 14 in all treatments, with the greatest decrease occurring in mixture replicate B for the steroid oestrogens. In contrast, the steroid oestrogens in mixture replicate A all increased. When combined, the steroid oestrogens produced an estimated EEQ (based on Young *et al.*, 2004), which also showed a good agreement between replicates and treatments.

Table 3.8 *Concentrations (ng/L) of anti-androgenic pharmaceuticals and steroid oestrogens in samples from treatment tanks collected on the first (d1) and final day (d14) of the experiment and quantified by LC-MS/MS. All test chemicals were below the LOD in the control and solvent control tanks*

Treatment	Nominal (ng/L)	Measured concentration (ng/L)		% of nominal	
		d1	d14	d1	d14
E1					
Steroid Oestrogens A	9.6	11.1	9.5	116%	99%
Steroid Oestrogens B	9.6	10.0	7.3	104%	76%
Mixture A	9.6	8.5	12.8	88%	133%
Mixture B	9.6	10.1	3.9	105%	41%
E2					
Steroid Oestrogens A	2.1	2.7	1.2	130%	59%
Steroid Oestrogens B	2.1	2.1	1.3	100%	60%
Mixture A	2.1	1.9	2.1	90%	101%
Mixture B	2.1	2.0	0.7	96%	32%
EE2					
Steroid Oestrogens A	0.3	0.3	0.2	113%	69%
Steroid Oestrogens B	0.3	0.3	0.2	94%	61%
Mixture A	0.3	0.2	0.3	81%	110%
Mixture B	0.3	0.2	0.1	78%	33%
Positive Control A	10	11.6	7.7	116%	77%
Positive Control B	10	7.0	10.9	70%	109%
Bicalutamide					
Anti-androgen A	115	113	75	98%	65%
Anti-androgen B	115	166	76	144%	66%
Mixture A	115	114	73	99%	63%
Mixture B	115	102	77	89%	67%

	Nominal (ng/L)	**Predicted EEQ (ng/L)**		**% of nominal**	
		d1	**d14**	**d1**	**d14**
EEQ					
Steroid Oestrogens A	8.3	9.8	6.5	118%	78%
Steroid Oestrogens B	8.3	8.3	5.5	99%	67%
Mixture A	8.3	7.1	9.7	86%	117%
Mixture B	8.3	7.7	3.0	93%	36%
Positive Control A	100	116	77.1	116%	77%
Positive Control B	100	70.3	109	70%	109%

	Nominal (ng/L)	**Estimated CPA (ng/L)**		**% of nominal**	
		d1	**d14**	**d1**	**d14**
Cyproterone acetate					
Anti-androgen A	74	73	48	98%	65%
Anti-androgen B	74	107	49	144%	66%
Mixture A	74	73	47	99%	63%
Mixture B	74	66	50	89%	67%

3.3.1.2 Somatic growth, GSI and HSI

Differences in gross indices between treatment groups are displayed in Figure 3.7. In this experiment, fork length and weight did not vary between treatments, but statistically significant variance was found in the condition factor (one-way ANOVA, $F_{5,90}$ = 2.66, P ≤ 0.05). However, post hoc analysis found that the only significant difference between treatment groups occurred between the mixture treatment and the solvent control, where condition factor of fish in the mixture treatment was significantly higher. In general, a condition factor over 1 is considered to indicate good health and nutritional status in a fish. Only two fish (one in the solvent control and one in the steroid oestrogen treatment) were found to have condition factors below 1, suggesting that the general health of the exposure population was good. In fact, with the exception of the single fish with a low condition factor (0.8) the solvent control values ranged from 1.4-2.0 in comparison to 1.5-2.1 in the mixture treatment.

For reproductively active male fathead minnows, GSI has been reported as around 1-2% of total body weight (Watanabe *et al.*, 2007; Jensen *et al.*, 2001). This study found higher GSI in all treatment groups with the solvent control fish ranging from 0.4-3.6%, which varied significantly between treatment groups (one-way ANOVA, $F_{5,90}$ = 4.38, P ≤ 0.001). However, the GSI of fish in the solvent control was not significantly different from those in the other treatment tanks, although there were significant differences between anti-androgen, steroid oestrogen and mixture treatment groups. In contrast, HSI was not found to significantly vary between treatment groups.

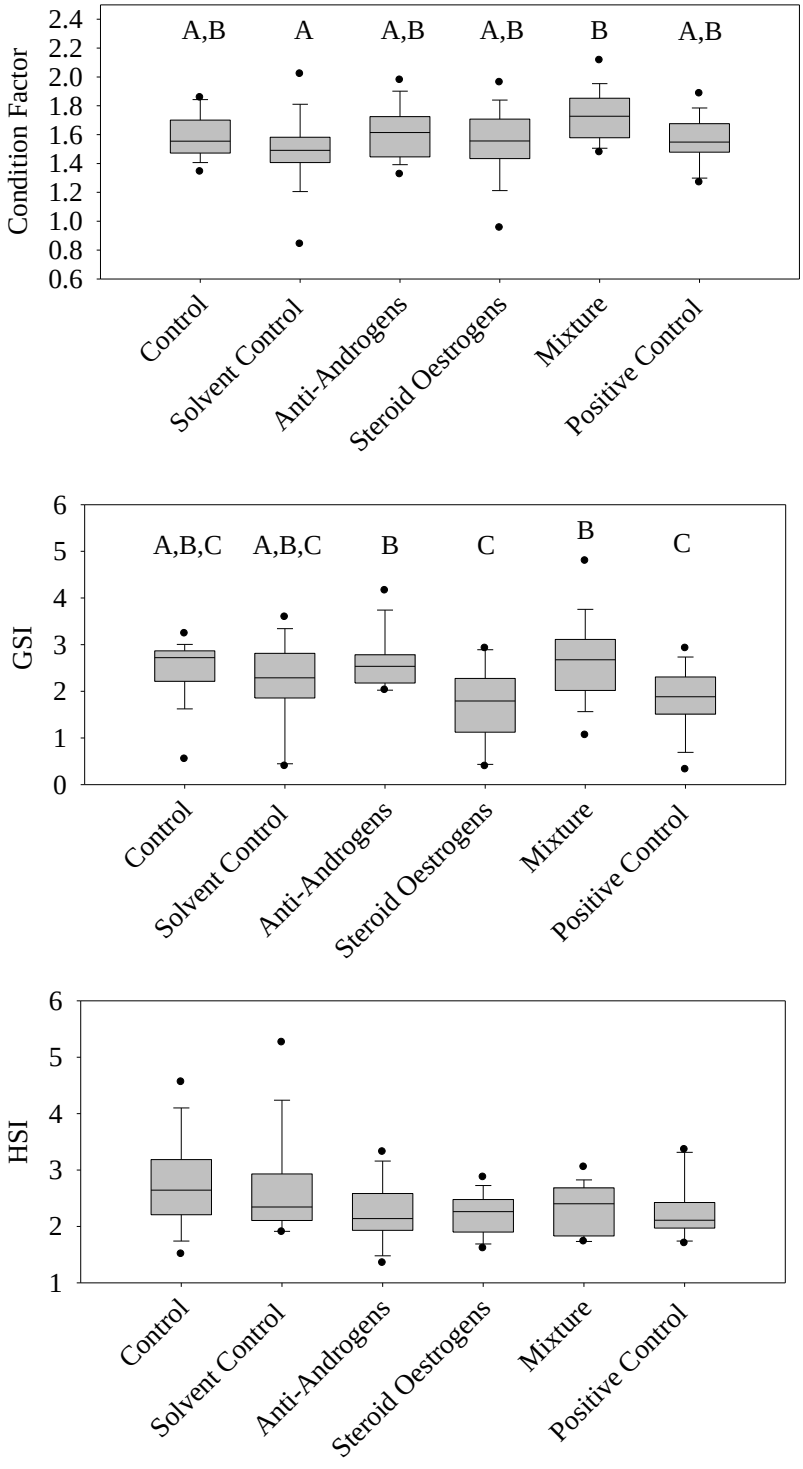


Figure 3.7 *The condition factor, GSI and HSI of fathead minnows in each treatment group (N=16). Boxes represent the median with 25th and 75th percentiles with error bars extending to the 95th percentile and outliers shown as dots. A,B and C denote statistically significant differences between groups at p≤0.05.*

3.3.1.3 Secondary sexual characteristics

In this experiment, nuptial tubercles and fatpads were observed in all fish from all treatments (Figure 3.8). No treatment effects were observed for the fatpad index, which did not significantly vary between treatment groups. However, both tubercle number and prominence were found to vary significantly (tubercle number: one-way ANOVA, $F_{5,90}$ = 6.63, P ≤ 0.001; tubercle prominence: Kruskall-Wallis one-way ANOVA on ranks, P ≤ 0.001). Post hoc analysis found that the carrier solvent did not affect these endpoints and no significant effect of anti-androgenic pharmaceuticals, steroid oestrogens or the mixture treatments were detected in comparison to the solvent control. However, both characteristics were significantly lower in the positive control.

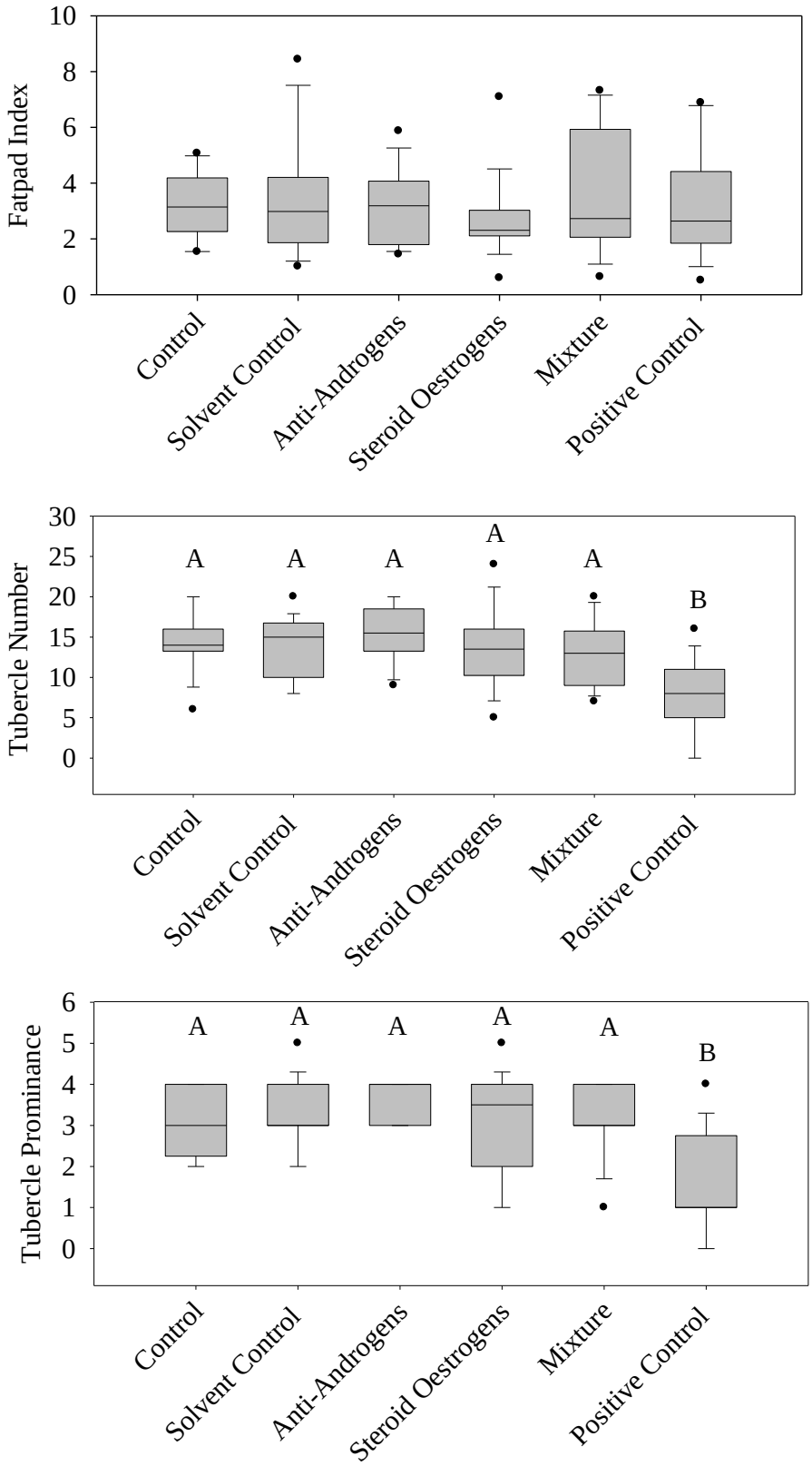


Figure 3.8 *Secondary sexual characteristics of fathead minnows in each treatment group (N=8). Boxes represent the median with 25th and 75th percentiles with error bars extending to the 95th percentile and outliers shown as dots. A and B denote statistically significant differences between groups at p≤0.05.*

3.3.1.4 Vitellogenin induction

Vitellogenin concentrations were measured in blood plasma samples from male fathead minnowss by ELISA. Although there were 16 fish employed in each treatment, only individuals whose VTG measurements were within the working range of the test kit were included in statistical analysis. The number of samples (*n*) is indicated in Figure 3.9 below. Statistical analysis found that vitellogenin concentrations varied significantly between treatments (Kruskall-Wallis one-way ANOVA, $P \leq 0.001$, Figure 3.9). No significant induction was observed in the solvent control, which was statistically similar to the control with a median plasma vitellogenin concentration of 76 (8-3,855) ng/mL. These were comparable with the lower end scores for unexposed fathead minnows reported in a historical control database, where the median plasma vitellogenin was recorded as 2,500 ng/mL (Watanabe *et al.*, 2007). The greatest induction was observed in the positive control, where the fish had a median plasma concentration of 26 (13-50) mg/mL. This did not significantly differ from the oestrogen or mixture treatments. Fish treated with the anti-androgenic pharmaceuticals alone had a median vitellogenin concentration of 111 (4-3,223) ng/mL, 1.5 fold higher than the solvent control, but still statistically similar. In contrast, the steroid oestrogens caused a significantly greater induction than the anti-androgens alone, with a fold increase of around 30,000, based on the median plasma concentration of 2.5 (0.02-7) mg/mL. A 100,000 fold increase was observed in the mixture treatment, which had a median concentration of 8 mg/mL, although the range of concentrations was more variable (0.03-27 mg/mL) due to the low concentration response of one fish. Nonetheless, vitellogenin concentrations in fish in the mixture treatment did not differ significantly from the oestrogens alone. Statistical analysis was repeated with the outlier in the mixture treatment discounted, to assess whether statistical differences between the oestrogen and mixture treatments would occur. However, the results were no different from the original analysis. When the induction of vitellogenin was compared with the historical control database, the induction observed in the oestrogen and mixture treatments compared well with the highest concentrations observed in normal female fish (median 16.5 mg/mL, maximum 47.5 mg/mL) (Watanabe *et al.*, 2007).

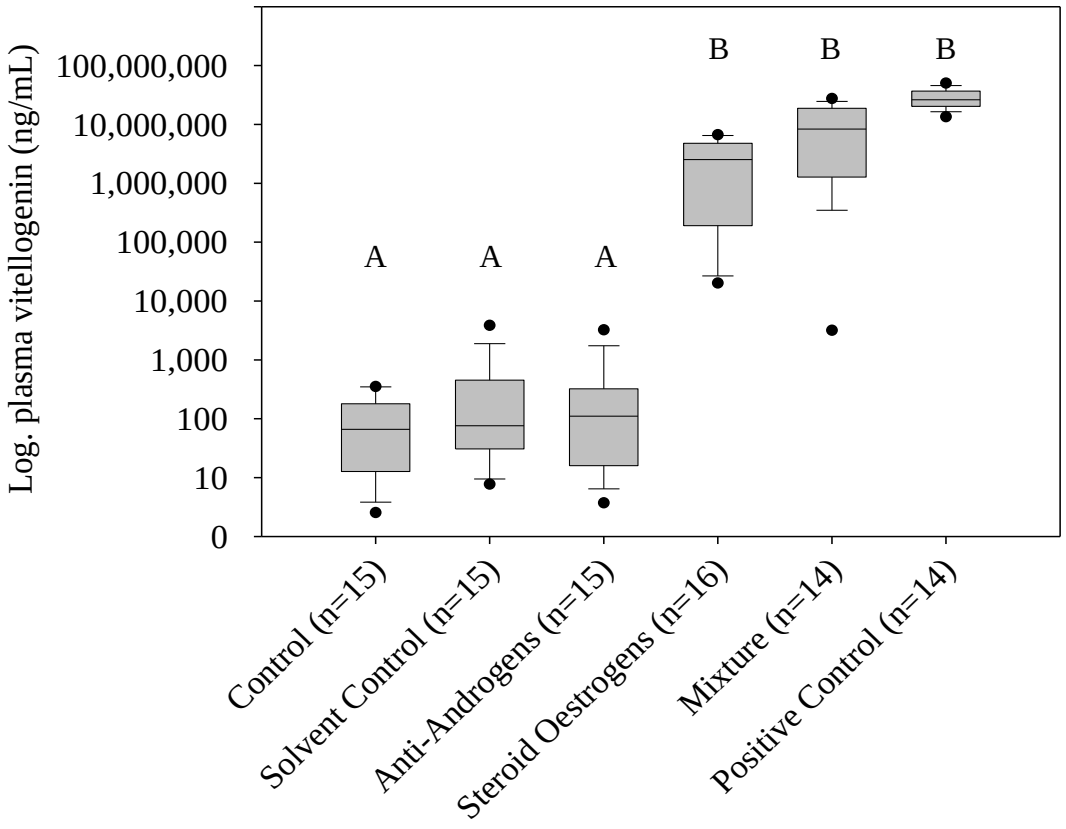


Figure 3.9 *Log. vitellogenin concentrations (ng/mL) in the blood plasma of fathead minnows in each treatment group quantified by ELISA. The number of samples accurately quantified and displayed for each treatment group is indicated (n). Boxes represent the median with 25th and 75th percentiles with error bars extending to the 95th percentile and outliers shown as dots. A and B denote statistically significant differences between groups at p≤0.05.*

3.3.2 Experiment two

In this experiment, Japanese medaka were exposed from an embryonic to early adult stage to mixtures of anti-androgenic pharmaceuticals and steroid oestrogens in singular and combined activity mixture treatments. Two doses were employed using concentrations with environmental relevance to effluent and river concentrations, produced by predictive modelling.

3.3.2.1 Chemical analysis

Aquarium water samples were collected from all treatment tanks at four time points throughout the experimental duration for detection and quantification of the chemicals. However, in some cases samples were discounted due to unreliable, variable detection of the internal standards, which affected quantification. This was a particular issue with cyproterone acetate where quantification of d_{10} carbamazepine was highly variable, which led to the data being discounted. Nonetheless, cyproterone acetate was identified as being present only in the appropriate tanks, with peaks exceeding three times the signal to noise ratio. Furthermore, the extraction of only a 100 mL sample was insufficient to accurately detect EE2. As a result, the concentrations of cyproterone acetate and EE2 were estimated based on the expected ratios to E2 and bicalutamide respectively. A summary of these data are shown in Table 3.9.

Table 3.9 *Concentrations of anti-androgenic pharmaceuticals and steroid oestrogens in treatment tanks compared to their nominal concentrations. Concentrations of EE2 and cyproterone acetate were estimated based on their expected ratios to E2 and bicalutamide respectively. n shows the number of samples analysed over the duration of the experiment.*

		E1 (ng/L)				E2 (ng/L)			
Treatment	**Replicate**	Nominal (ng/L)	n	Mean measured (min-max)	% of nominal	Nominal (ng/L)	n	Mean measured (min-max)	% of nominal
Control	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
Solvent Control	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
Low Anti-androgen	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
Low Oestrogen	A	13	4	24 (20-30)	186%	2	4	3.2 (2.3-4.3)	160%
	B	13	4	27 (17-35)	204%	2	4	3.4 (2.3-4.3)	172%
Low Mixture	A	13	3	33 (25-44)	253%	2	4	3.9 (2.3-6.2)	196%
	B	13	3	34 (24-39)	258%	2	4	3.7 (1.9-5.4)	183%
High Anti-androgen	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
High Oestrogen	A	29	4	63 (59-68)	217%	4	4	8.3 (6.3-9.8)	206%
	B	29	4	66 (57-88)	229%	4	4	6.8 (6.1-8.1)	171%
High Mixture	A	29	3	64 (45-82)	220%	4	4	8.7 (6.6-12.5)	216%
	B	29	4	61 (43-75)	211%	4	4	9.5 (7.2-16.2)	238%
Positive Control	A	0	4	3 (2-4)		50	4	76 (64-86)	152%
	B	0	4	3 (<LOD-6)		50	4	80 (52-96)	160%

Table 3.9 continued…

Treatment	Replicate	Estimated EE2 (ng/L)				Predicted EEQ (ng/L)			
		Nominal (ng/L)	n	Mean (min-max)	% of nominal	Nominal (ng/L)	n	Mean (min-max)	% of nominal
Control	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
Solvent Control	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
Low Anti-androgen	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
Low Oestrogen	A	0.2	4	0.3 (0.2-0.4)	160%	8	4	14 (12-17)	174%
	B	0.2	4	0.3 (0.2-0.4)	172%	8	4	16 (10-19)	189%
Low Mixture	A	0.2	4	0.4 (0.2-0.6)	196%	8	3	20 (16-23)	239%
	B	0.2	4	0.4 (0.2-0.5)	183%	8	3	20 (17-24)	236%
High Anti-androgen	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
High Oestrogen	A	0.4	4	0.8 (0.6-1.0)	206%	18	4	37 (32-40)	212%
	B	0.4	4	0.7 (0.6-0.8)	171%	18	4	36 (32-46)	203%
High Mixture	A	0.4	3	0.9 (0.7-1.3)	216%	18	3	39 (34-41)	216%
	B	0.4	4	1.0 (0.7-1.6)	238%	18	4	39 (35-47)	223%
Positive Control	A	0	4	<LOD		50	4	77 (66-87)	154%
	B	0	4	<LOD		50	4	81 (52-98)	162%

Treatment	Replicate	Bicalutamide (ng/L)				Estimated Cyproterone acetate (ng/L)			
		Nominal (ng/L)	n	Mean measured (min-max)	% of nominal	Nominal (ng/L)	n	Mean measured (min-max)	% of nominal
Control	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
Solvent Control	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
Low Anti-androgen	A	53	3	99 (80-135)	187%	32	3	57 (48-82)	178%
	B	53	4	71 (51-82)	134%	32	4	43 (31-50)	134%
Low Oestrogen	A	0	4	<LOD		0	4	<LOD	
	B		4	<LOD		0	4	<LOD	
Low Mixture	A	53	3	107 (96-118)	201%	32	3	64 (58-71)	201%
	B	53	3	85 (56-112)	161%	32	3	52 (34-68)	161%
High Anti-androgen	A	115	2	133 (119-146)	115%	74	2	85 (77-94)	115%
	B	115	3	152 (136-172)	132%	74	3	98 (88-111)	132%
High Oestrogen	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	
High Mixture	A	115	2	99 (69-129)	86%	74	2	64 (44-83)	86%
	B	115	3	134 (124-139)	117%	74	3	86 (80-89)	117%
Positive Control	A	0	4	<LOD		0	4	<LOD	
	B	0	4	<LOD		0	4	<LOD	

The degradation of the exposure chemicals in 100 mL of MFS water over 24 hours was first assessed to determine the probable conditions during petri dish dosing at the critical stages of early development. Following extraction, immediately after spiking the water sample, concentrations of 23 ng/L E1, 2.0 ng/L E2 and 149 ng/L bicalutamide were detected. These were all slightly above the nominal concentrations with the

exception of E2. After 24 hours, concentrations were 22 ng/L E1, 1.4 ng/L E2 and 115 ng/L bicalutamide. This suggests that the exposure concentrations were maintained to an acceptable degree by the daily stock renewal. When tank water was sampled during the latter part of the experiment, no contamination of control or solvent control tanks was detected and no incidence of cross contamination between oestrogen and anti-androgen treatment tanks was observed. For E1 and E2, concentrations varied and exceeded the nominal throughout the study by up to 158% for E1 and 138% for E2. It was estimated that this would be similar for EE2. Nonetheless, good agreement was achieved between replicate tanks and between oestrogen and mixture treatment tanks within the same dosing regimen (low or high). In addition, there was little overlap in the range of concentrations detected in the low and high treatments, which were clearly differentiated (demonstrated for the EEQ in Figure 3.10). Similar results were also found when the measured concentrations of E1 and E2 were combined with the estimated concentration of EE2 to determine the EEQ (based on Young *et al.*, 2004)**.**

Bicalutamide was successfully quantified in fewer samples than the steroid oestrogens; in some cases only twice out of the four samples. It also exceeded the nominal by up to 101%, less than the steroid oestrogens in some cases even though they were dosed from the same stocks in the mixture treatments. The differences between replicates tended to be greater than the steroid oestrogens. In addition, there was greater crossover between the two doses, as shown in Figure 3.10. The low doses ranged from 51-135 ng/L, whilst the high doses ranged from 69-172 ng/L where one of the samples in the high mixture A dropped below the nominal. The situation was expected to be similar for cyproterone acetate, since it was dosed alongside bicalutamide in all treatments and was analysed by LC-MS/MS at a similar time. Here, concentrations of 31-82 ng/L were predicted to occur in the low doses in comparison to 44-111 ng/L in the high doses.

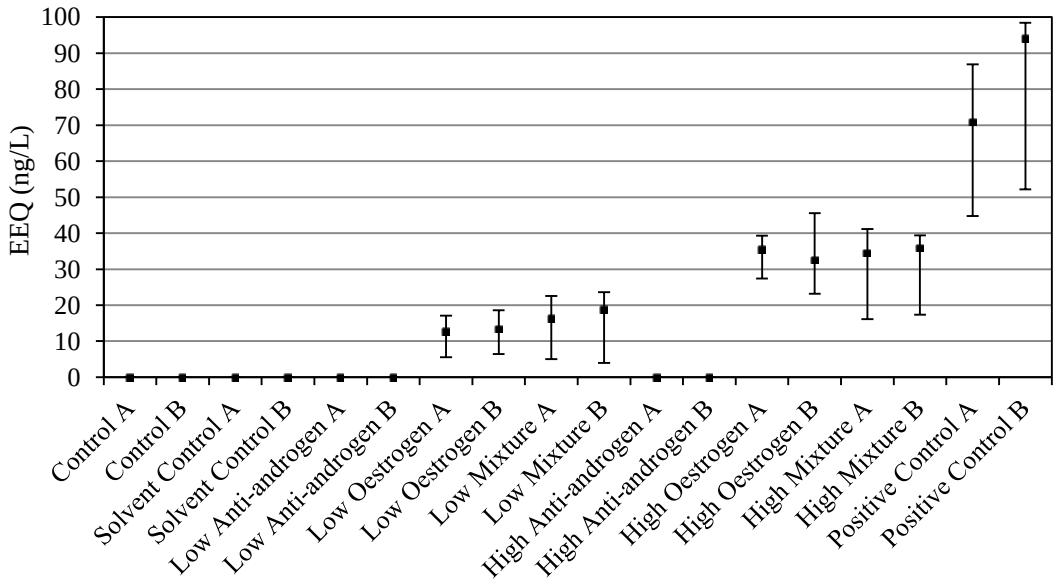


Figure 3.10 ***(A)*** *the combined E2 equivalent (EEQ) of water samples from experiment two determined from the measured E1 and E2 concentrations and the predicted EE2 concentrations* (based on Young *et al.*, 2004)***.*** *A and B refer to replicate tanks.*

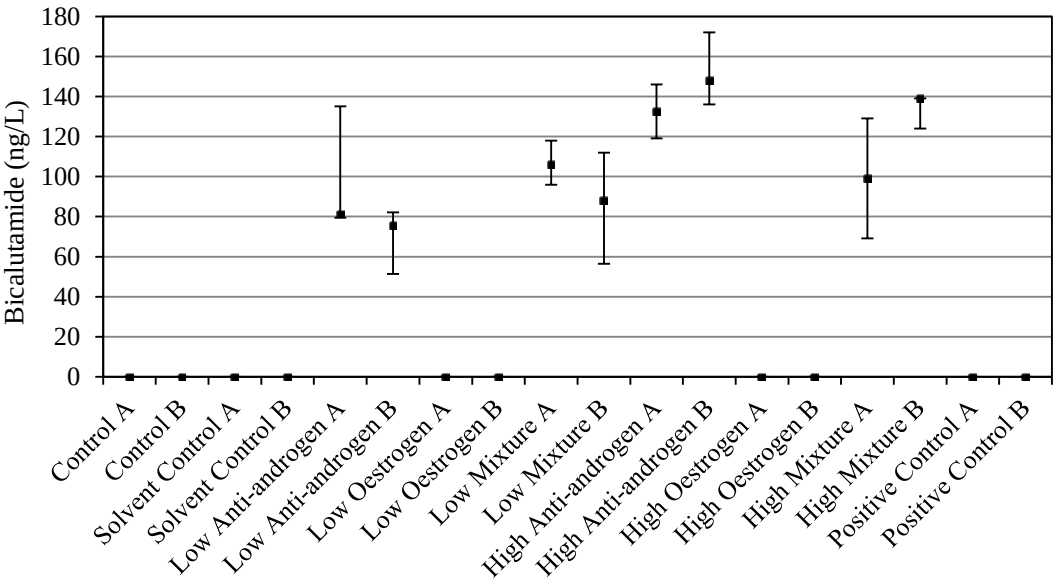


Figure 3.10 ***(B)*** *The measured concentrations of bicalutamide. The median concentrations are shown with error bars extending to the minimum and maximum. A and B refer to replicate tanks.*

3.3.2.2 Survival

In most tanks, survival exceeded 80% following 98 days exposure (Table 3.10). This was considerably lower in the positive control (64% treatment survival), which led to the decision to sample the fish early following 90 days exposure. Lower survival was also observed in control tank A and solvent control tank A. In addition, there were 27 fish in the low anti-androgen treatment B that survived until the end of the experiment, suggesting that there was a miscount in the number of embryos employed at the start of the experiment.

Table 3.10 *The survival of fish in the replicate tanks from each treatment group. Each tank started with 25 embryos.*

Tank		n	% Replicate Survival	% Treatment Survival
Control	A	14	56%	78%
	B	25	100%	
Solvent Control	A	18	72%	76%
	B	20	80%	
Low Anti-androgens	A	20	80%	94%
	B	27	108%	
Low Steroid Oestrogens	A	23	92%	92%
	B	23	92%	
Low Mixture	A	24	96%	96%
	B	24	96%	
High Anti-androgen	A	20	80%	88%
	B	24	96%	
High Steroid Oestrogens	A	23	92%	92%
	B	23	92%	
High Mixture	A	23	92%	88%
	B	21	84%	
Positive Control	A	15	60%	64%
	B	17	68%	

3.3.2.3 Somatic growth

Both the length and weight of fish significantly varied between treatment groups (Kruskall-Wallis one-way ANOVA, $P \leq 0.001$). Interestingly, the fish in the high mixture treatment were significantly longer and heavier than the solvent control and the high anti-androgen treatment alone (Figure 3.11). However, these did not differ from the

high steroid oestrogen treatment, for which only the length was significantly higher than the solvent control. Condition factor was also shown to significantly vary amongst treatments (Kruskall-Wallis one-way ANOVA, $P \leq 0.001$). However, post hoc analysis found that neither doses of the anti-androgenic pharmaceuticals or steroid oestrogens alone, or in the combined mixture, caused any effect. In contrast, condition factor in the positive control was significantly higher than all other treatments. In addition, in the control, high anti-androgen and positive control treatments, some fish experienced very low growth. They did not appear to have progressed from a fry stage and were below 20 or even 10 mm in length. In comparison, the median lengths in all treatments exceeded 30 mm.

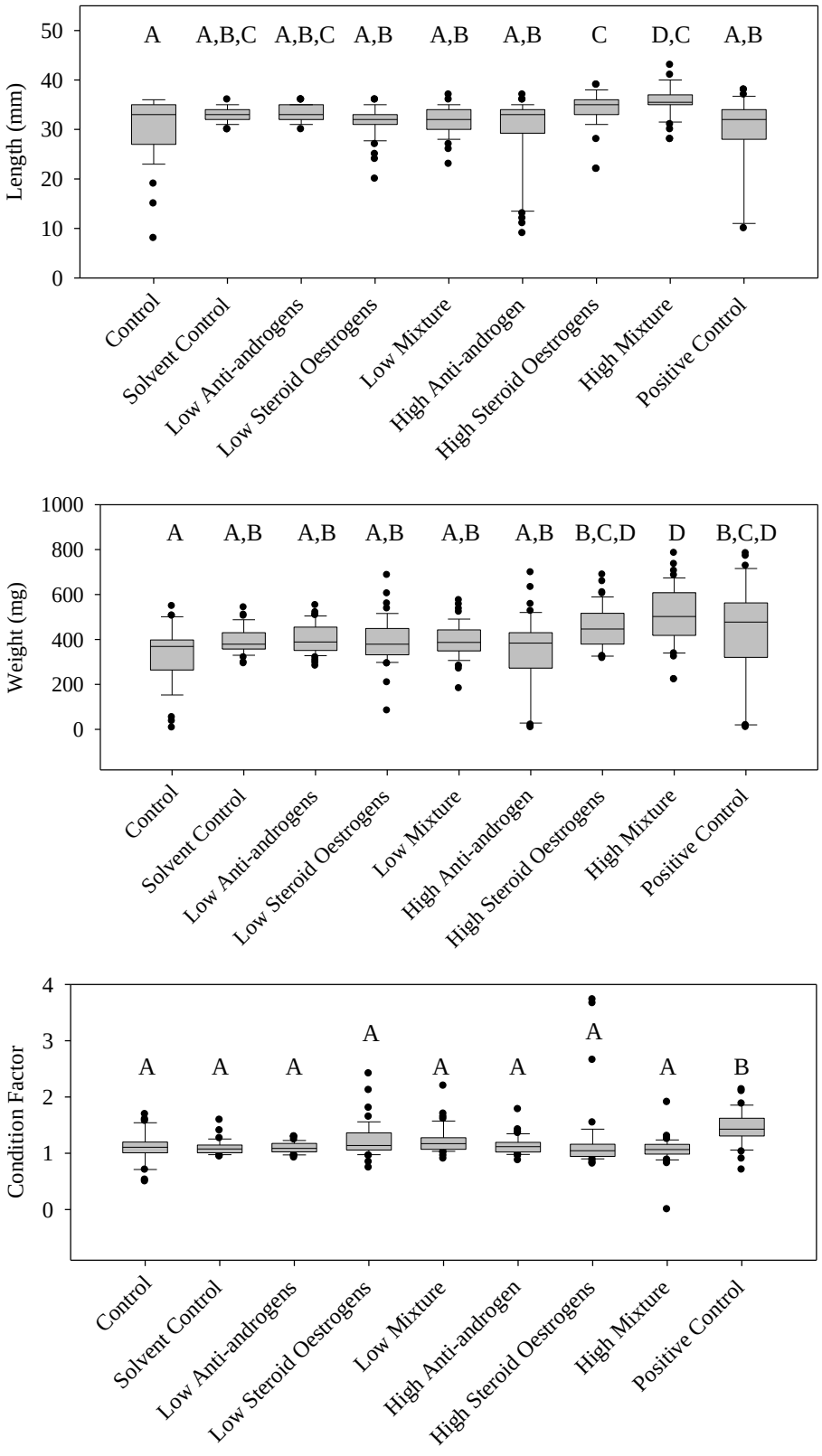


Figure 3.11 *Length, weight and condition factor of fish in each treatment group. Boxes represent the median with 25th and 75th percentiles with error bars extending to the 95th percentile and outliers shown as dots. A,B,C and D denote statistically significant differences between groups at p≤0.05.*

3.3.2.4 Sex ratio and intersex induction

An intriguing result which became apparent during sampling was the excessive number of phenotypically male fish in the treatment groups. In fact, both the control and solvent control treatments appeared to be made up exclusively of male fish, in contrast to the positive control, where all fish appeared to be female. In fact, only a small number of females were identified in the other treatment groups (Figure 3.12). This unexpected result was confirmed with gonad histology, where females were confirmed. In a few fish in the control, high anti-androgen and positive control treatments, sex could not be identified. In these cases, the fish tended to be those that had failed to develop to an adult stage and their gonads were undifferentiated or not identified by the histological method.

Intersex was defined only by the presence of oocytes in testicular tissue since ovarian cavities could not be properly quantified in the longitudinal sections. Treatment of medaka with steroid oestrogens alone caused intersex induction in 22% of the treated population for the low dose and 70% of those treated with the high dose respectively (Figure 3.12). However, only the high oestrogen treatment induced what was considered a significant induction of intersex in comparison to the solvent control (Williams contrast test $p \leq 0.0001$), where intersex was identified in 6% of the treatment population. A small proportion of fish in the low anti-androgen treatment were also found to be intersex (6%), which did not significantly differ from the solvent control and no intersex fish were found in the high anti-androgen treatment. Interestingly, in both the low and high mixture treatments, the number of intersex individuals was 9% and 10% higher, respectively, in comparison to their corresponding oestrogen treatments alone. However, whilst intersex incidence in these treatments was significantly higher than the solvent control (Williams contrast test: low treatment $p \leq 0.01$; high treatment $p \leq 0.001$), there was no significant difference between the mixture treatments and their respective oestrogen treatments.

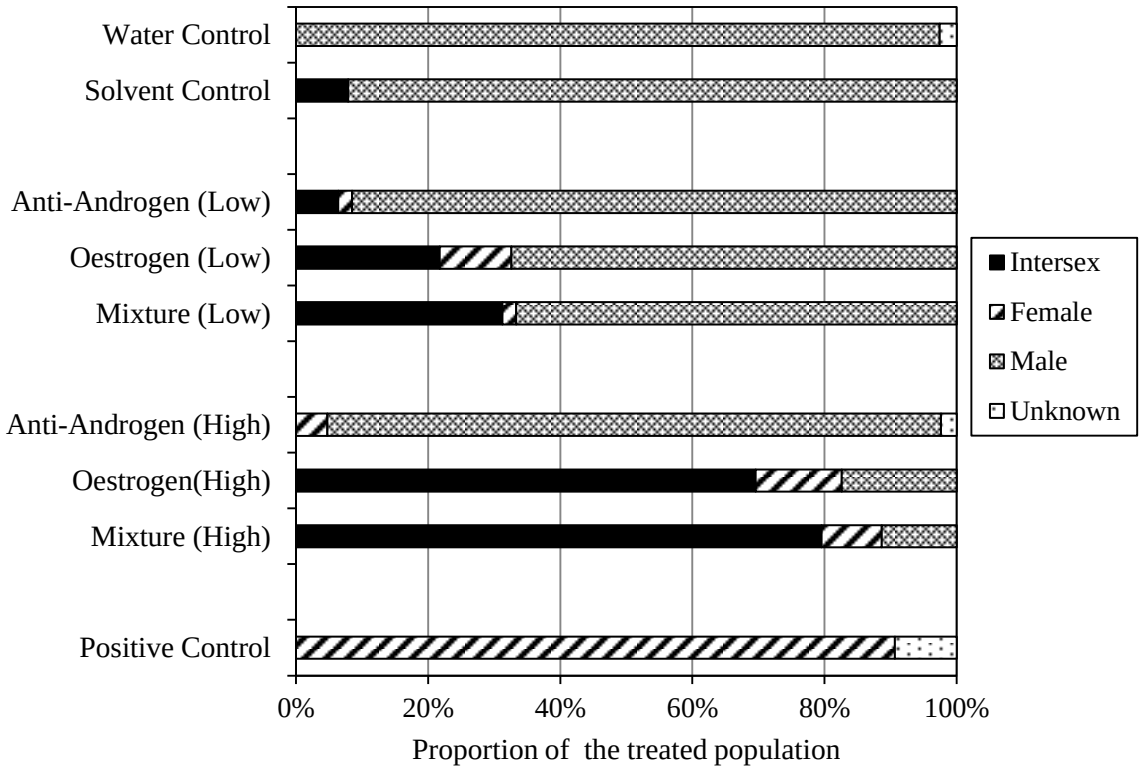


Figure 3.12 *The proportions of male, female, intersex and unknown Japanese medaka within each treatment group.*

3.3.2.5 Intersex severity

Intersex severity was scored for each fish based on the maximum intersex index (scored 1-6) identified in gonadal sections (Table 3.6). Images of histological characteristics typical to some of the treatments are shown in Figure 3.13. Statistical analysis of severity between treatment groups is displayed in Figure 3.14, in which a comparison with a modelled odds ratio and its 95% confidence limits exceeding 1 is determined as statistically significant.

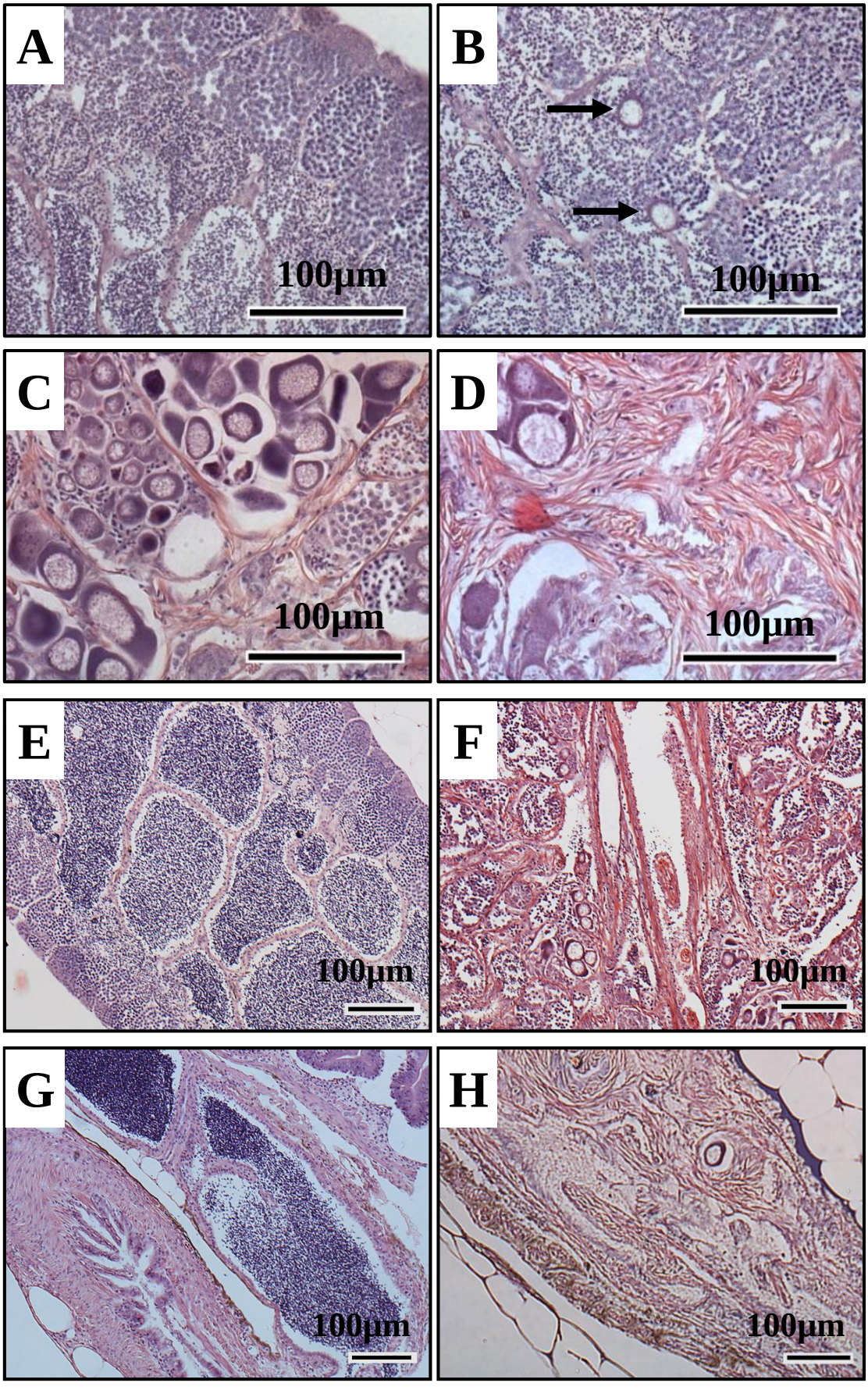

A
100µm
B
100µm
C
100µm
D
100µm
E
100µm
F
100µm
G
100µm
H
100µm

Figure 3.13 *Images showing the gonad histopathology of fish sampled during this experiment. (A) A typical gonad of a male from the solvent control. (B) Sporadic oocytes (shown with arrows) in the testes typical of the low oestrogen/mixture treatments (intersex index = 1). (C) The increased abundance and clustering of oocytes in the testes experienced in the high oestrogen/mixture treatment (intersex index = 6). (D) Extensive fibrosis which occurred in the testes of some high oestrogen and high mixture treated fish, in some casing taking up the entire gonad structure. (E) Connective tissue forming testicular lobules containing spermatozoa in a central efferent duct from a control fish compared with the disorganisation of lobule and duct formation in a fish from the high mixture treatment (F). (G) The sperm duct from a control fish containing high numbers of spermatozoa in comparison to the sperm duct of a fish from the high mixture which was empty apart from a single primary oocyte (H).*

Control fish showed healthy, lobulated gonads in the advanced stages of spermatogenesis, with high numbers of spermatozoa in the lumen and sperm ducts (Figure 3.13 A, E and G). This was also observed in the solvent controls, although a low incidence of intersex was observed in this treatment which was characterised by very few, sporadic primary oocytes (index 1). This was similar in the low anti-androgen treatment. In comparison, most intersex fish in the low oestrogen treatment also had an intersex index of 1 (Figure 3.13 B), although three fish scored higher with an intersex index of 6 occurring in two fish, where the oocytes were numerous and clustered. Unlike intersex incidence, intersex severity in fish from the low oestrogen treatment was significantly higher than those in the solvent control based on the odds ratios (Figure 3.14). In the low mixture treatment, more fish were identified with an intersex index over 1 than the oestrogen treatment alone (47% and 30% respectively). However, there was no statistically significant difference between these groups based on the odds ratio.

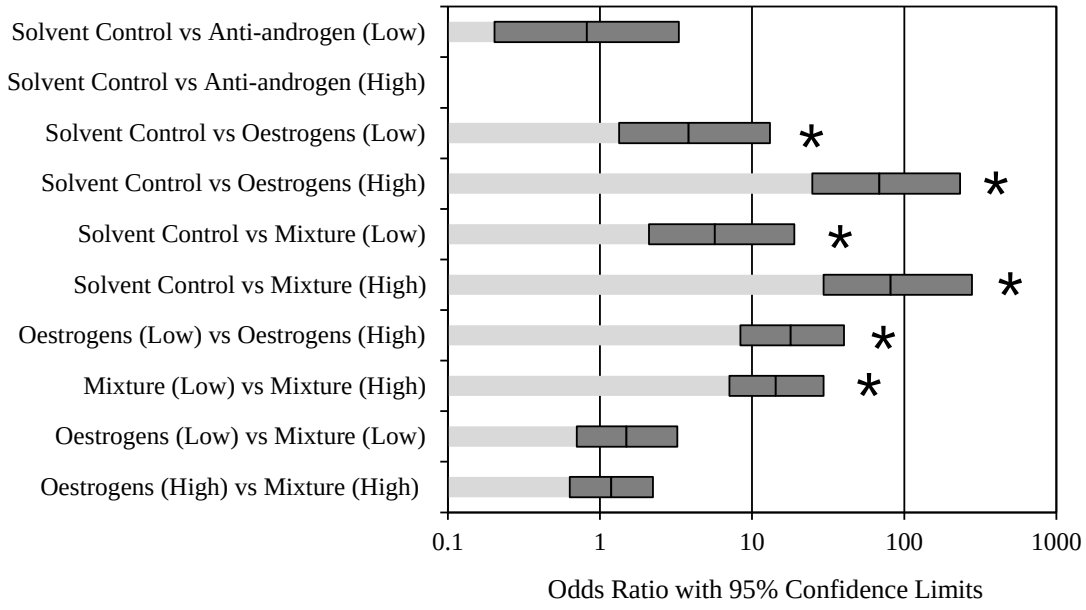


Figure 3.14 *Statistical comparisons of the intersex severity in Japanese medaka between treatment groups. The boxes represent the odds ratio and extend to the 95% confidence limits where a significant difference between groups is judged when the boxes exceed the odds ratio of 1. Significantly different comparisons are indicated by an asterisk*. *No comparison was made between the solvent control and the high anti-androgen treatment, since no intersex fish were identified.*

Although intersex fish were identified in the low anti-androgen treatment, none were found in the high anti-androgen treatment, where the gonads still appeared to be similar to the controls and solvent controls. In contrast, intersex incidence and severity significantly increased in both the high oestrogen and the high mixture treatments in comparison to the low doses (Figure 3.14). In these treatments, intersex index in the gonadal sections still varied from 1-6. However, there was an increased proportion with a higher index, characterised by large clusters of oocytes, which were separated from the testicular tissue (Figure 3.13 C). In the more severe cases, oocytes made up the majority of the gonad tissue and a few secondary oocytes were also observed in two fish from the high mixture treatment and one fish from the high oestrogen treatment. In addition, increased lobular disorganisation was observed in both intersex and non-intersex males from these treatments (Figure 3.13 F). Fibrotic tissue was also identified and in some cases it was severe enough to make up a majority of the gonad tissue in the high oestrogen and mixture treatments. An example is shown in Figure 3.13 D and

H, and further images are available in the Appendix (Figure A2.1). Due to the lack of functional testicular tissue in some cases where oocytes and extensive fibrotic tissue occurred, these sections were classified as index 6 in statistical analysis for severity. This was due to the lack of testicular tissue, which indicated a high level of gonadal malformation similar to that of individuals with a high proportion of ovarian tissue. In addition, fish within the high oestrogen and high mixture treatments showed reductions in spermatozoa in the sperm ducts in comparison to the solvent control and in some fish, particularly those with extensive fibrosis, the ducts were empty (Figure 3.13 H). When statistical analysis was employed, the odds ratio did not show a statistically significant increase in the overall intersex severity in the high mixture treatment in comparison to the high oestrogen treatment. In the positive control, the fish were found to be histologically female, with the exception of a few smaller fish where the gonads remained unidentified. Large, mature ovaries were identified where multiple stages of oogenesis were observed, which included vitellogenic, secondary oocytes. No testicular tissue was identified, although some contained interstitial proteinaceous fluid, which was likely to be vitellogenin. No obvious abnormalities were observed in phenotypically female fish in the positive control or any of the other treatments where they occurred.

3.3.3 UK catchment modelling of anti-androgenic pharmaceuticals

Subsequent hydrological modelling of river catchments in England and Wales with LF2000-WQX found that contamination with anti-androgenic pharmaceuticals is likely to be widespread, as indicated in Figure 3.15 and Figure 3.16. On these contaminated stretches, downstream of WWTW discharges, median concentrations of 9 (0.0002-157) ng/L bicalutamide and 5 (0.0001-86) ng/L of cyproterone acetate were predicted to occur. These exceeded the concentrations predicted on the River Erewash in chapter two of 26 (16-50) ng/L bicalutamide and 17 (10-32) ng/L cyproterone acetate, although the median concentration was lower. The five locations where the highest concentrations of anti-androgenic pharmaceuticals occurred were a mix of both rural and urban areas. The highest concentrations were achieved by effluent discharge into a tributary of the rural River Cole, Oxfordshire. Other stretches where concentrations exceeded 100 ng/L of bicalutamide were found on a tributary of the River Wye, Hertfordshire (urban), the Madford River, Devon (rural), the River Fos, Yorkshire (urban), a tributary to the River Ivel, Bedfordshire (urban) and Cuttle Brook, Oxfordshire (rural).

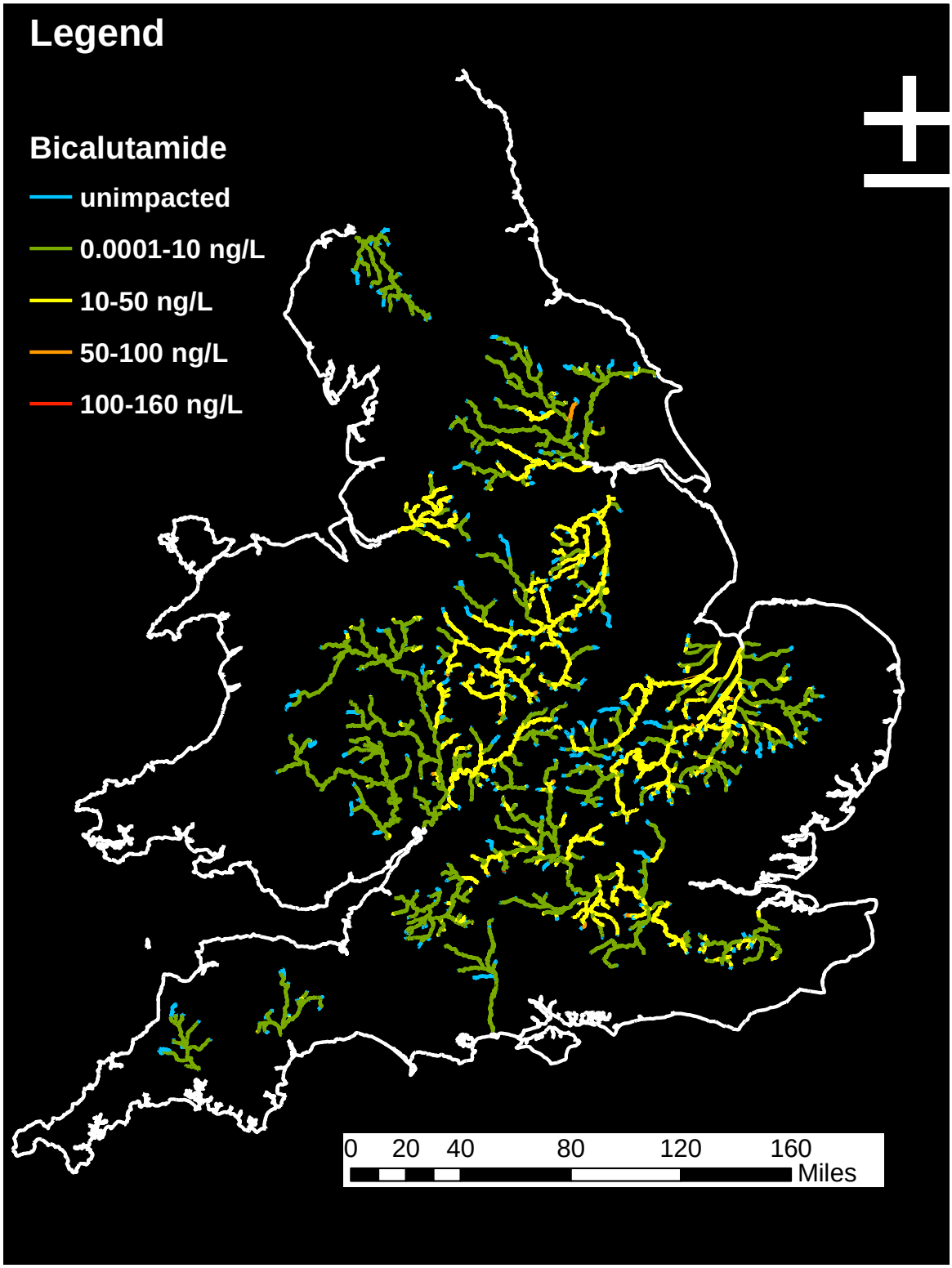


Figure 3.15 *A map showing the predicted concentrations of bicalutamide in river catchments in England and Wales based on 2009 prescriptions* (maps produced in ArcMap 10).

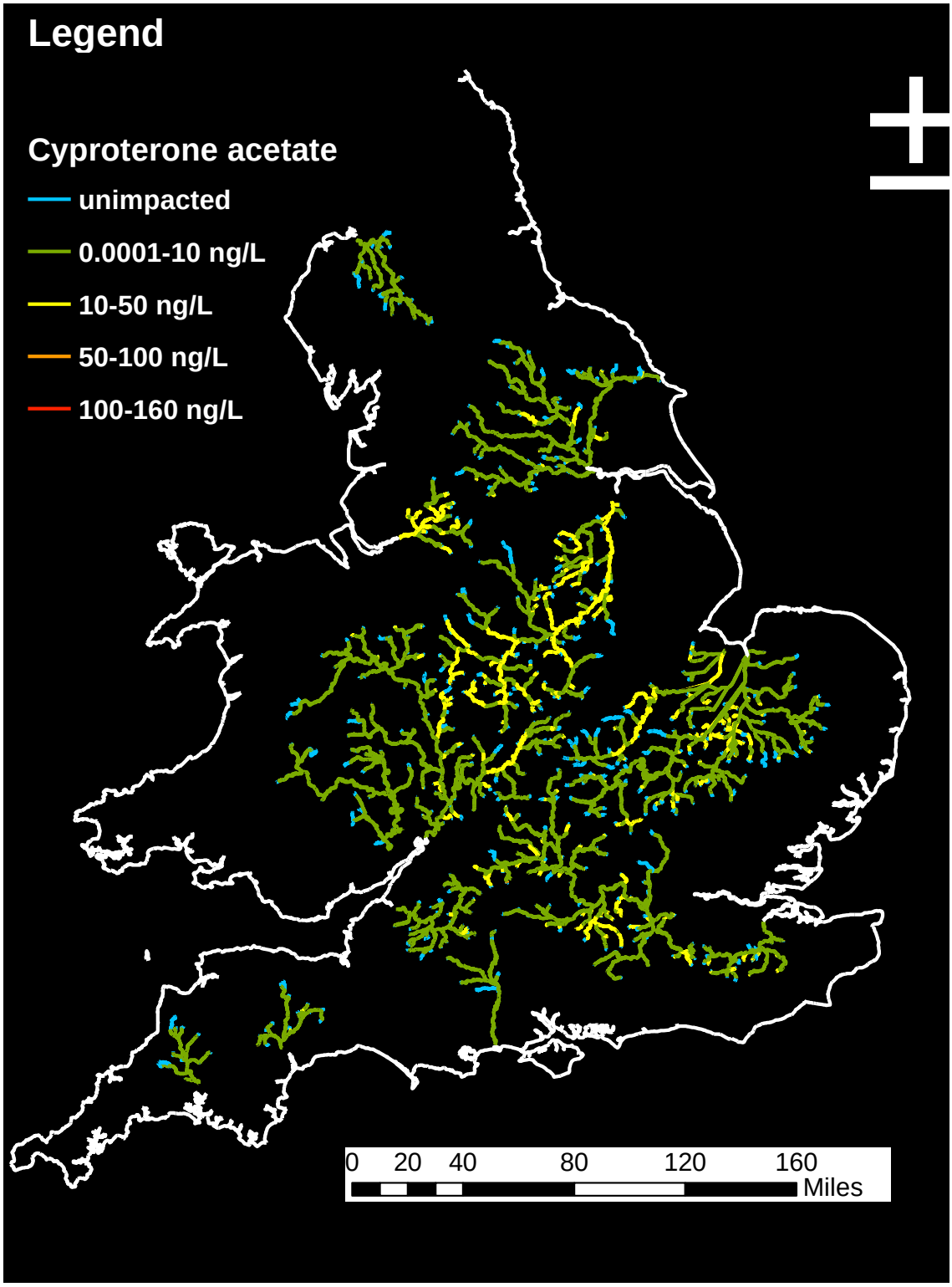


Figure 3.16 *A map showing the predicted concentrations of cyproterone acetate in river catchments in England and Wales based on 2009 prescriptions* (maps produced in ArcMap 10).

In experiments one and two, the measured concentrations of bicalutamide and those estimated for cyproterone acetate were generally found to be representative of the higher concentrations achieved on some river stretches. However, the predicted concentrations for the river catchments were exceeded on occasion in experiment one and in the high doses in experiment two. To recap, the maximum concentrations predicted to occur in the river catchments were 157 ng/L bicalutamide and 86 ng/L cyproterone acetate. In experiment one, bicalutamide was measured at 73-166 ng/L and cyproterone acetate was expected to be present at 47-107 ng/L. In this case, the predicted river concentrations were exceeded only once in the anti-androgen treatment A. Concentrations of bicalutamide predicted to occur in 30 of the modelled river stretches exceeded the lowest concentration measured in experiment one (73 ng/L), whilst 11 river stretches exceeded the 47 ng/L estimated for cyproterone acetate.

In experiment two, bicalutamide was measured between 69 and 172 ng/L in the high dose treatment, whilst 31-82 ng/L of cyproterone acetate was also estimated to be present. Again, the predicted river concentrations were exceeded only at one sampling point in the high anti-androgen treatment replicate B. The river concentrations in 41 river stretches exceeded the lowest concentration of bicalutamide measured in the high doses, in comparison to 16 stretches that were predicted to exceed cyproterone acetate. In the low dose treatments, the concentrations of anti-androgenic pharmaceuticals were within the range predicted to occur in the river catchments. Indeed, 115 river stretches exceeded the lowest measured concentration of bicalutamide (69 ng/L) and 87 stretches exceeded 31 ng/L cyproterone acetate.

3.4 Discussion

3.4.1 The environmental relevance of experimental concentrations

The concentrations of bicalutamide were analysed by LC-MS/MS and used to estimate the concentrations of cyproterone acetate present in treatment tanks. At the high doses, which were employed in both experiments one and two, the concentration range for these chemicals mostly remained within their predicted range for river catchments in England and Wales. However, this was occasionally exceeded, suggesting that the high dose anti-androgen concentrations employed in experiments one and two were more relevant to an effluent exposure scenario. In comparison, the low dose concentrations assessed in experiment two were all within the range of the predicted river concentrations and were therefore more likely to be relevant to exposure to high

river concentrations. Previous assessments by modelling and analytical measurement are few in number but compare well with the range predicted to occur in the modelled river catchments in this study. Indeed, up to 55 ng/L and 76 ng/L were reported for cyproterone acetate in effluent and river samples from the Langat River, Indonesia. However, it was hypothesised that additional input through improper disposal of the drug and outflow from pharmaceutical production facilities could have impacted these concentrations (Al-Odaini *et al.*, 2012). In addition, another study predicted that 10-40 ng/L of cyproterone acetate was likely to occur in effluent in France based on its prescription rates (Besse and Garric, 2009). Similar concentrations have also been predicted for bicalutamide as part of an environmental risk assessment, which anticipated concentrations of up to 59 ng/L could occur in surface water (AZ.com, 2013).

In the case of the steroid oestrogens, the concentrations measured by LC-MS/MS in experiment one were all within the range of concentrations measured in WWTW effluents (Johnson *et al.*, 2007b). They were also similar to the higher concentrations predicted to occur in river catchments in England and Wales (Williams *et al.*, 2009). In experiment two, the steroid oestrogen concentrations were measured at around 200% of the nominal. Nonetheless, these concentrations still supported the relevance of the high doses to effluent concentrations and the low doses to river concentrations (Williams *et al.*, 2009; Johnson *et al.*, 2007b). Although the concentrations exceeded the nominal, they are still likely to be an accurate depiction of the exposure scenario. This is likely to be due to issues in maintaining sufficient pressure in the header tanks during the study, which led to sporadic reductions in flow that decreased dilution in the treatment tanks to produce an exposure to higher concentrations than planned. The biological response in intersex induction to oestrogen exposure agreed with data from early life stage exposures of Japanese medaka to E2. At the low doses, the nominal EEQ was around 8 ng/L E2 equivalents, which would be expected to produce around 10% incidence of intersex, based on an early life exposure of medaka to 10 ng/L E2 (Metcalfe *et al.*, 2001). In fact the estimated EEQ in these treatments was 14-20 ng/L, which caused over 20% intersex in males. In addition, at the high doses where an EEQ of 36-39 ng/L was estimated to be present in the tanks, over 80% of males were intersex. This agrees well with Hirai et al., who induced 56% intersex in medaka sampled between 14 and 20 days post hatch following exposure to 33.5 ng/L E2 (Hirai *et al.*, 2006).

Based on these data it may be questioned why the anti-androgenic pharmaceuticals did not always occur at a similar percentage of the nominal to the steroid oestrogens in

experiment two, as they had done in experiment one. Due to the length of time between the analysis of the oestrogens and the anti-androgenic pharmaceuticals, degradation of samples is a possibility. In addition, the presence of fines in the samples could have caused further, differential loss of the chemicals. Nonetheless, if these chemicals occurred at 200% of nominal, the low doses would still be within the range of the predicted river concentrations from catchments in England and Wales. The high concentrations would also still have relevance to WWTW effluents, particularly given that the use of bicalutamide is projected to increase in the future based on the data presented in chapter two.

3.4.2 Experiment one: Vitellogenin and secondary sexual characteristics

A range of endpoints were assessed during this study which have been associated with sexual disruption in fish. Experiment one employed a 14 day exposure of juvenile male fathead minnows and assessed gross indices, secondary sexual characteristics and vitellogenin induction. This experimental set up has previously been used successfully to evaluate the effect of oestrogenic endocrine disruptors on vitellogenin induction, in single chemical and mixture studies (Brian *et al.*, 2005; Panter *et al.*, 2002). The lack of effect of anti-androgens on GSI in fathead minnows in this experiment was not unexpected as results from previous studies have also found no effect of anti-androgens on this endpoint. Indeed, flutamide, vinclozolin, *p,p*'-DDE and cyproterone acetate have failed to produce effects at higher concentrations than those used in this study (Hatef *et al.*, 2012; Filby *et al.*, 2007b; Panter *et al.*, 2004; Sharpe *et al.*, 2004; Mills *et al.*, 2001). However, a combination of oestrogenic octylphenol and anti-androgenic *p,p*'-DDE did significantly reduce GSI, but this was not assessed in comparison to single chemical treatments (Mills *et al.*, 2001). Steroid oestrogens have been shown to affect GSI in fish (Filby *et al.*, 2007b; Van den Belt *et al.*, 2003), but no effect was observed in either the steroid oestrogen or positive control treatments in this study. This could be due to the length of exposure since Filby *et al.* found a reduction in GSI in male fish exposed to 10 ng/L EE2 after 21 days in comparison to our 14 day duration.

The GSI, HSI and condition factor of the fish in this experiment were all high in comparison to a historical control database produced by the US Environmental Protection Agency (Watanabe *et al.*, 2007; Jensen *et al.*, 2001). This is likely to be reflective of the good nutritional status of fish held under our laboratory conditions. Indeed, HSI provides an indication of excess energy storage in the liver and GSI has

been shown to be higher when nutrient availability is not limited (Pope *et al.*, 2007; Plante *et al.*, 2005). Like GSI, no effect of anti-androgenic pharmaceuticals or steroid oestrogens was reflected in the HSI, which is of particular interest since the liver is also the site of vitellogenin production. Indeed, increases in HSI have been previously reported in oestrogen treated fish (Zha *et al.*, 2007; Purdom *et al.*, 1994), although the anti-androgen vinclozolin produced no effect in goldfish (Hatef *et al.*, 2012). Whilst useful as an initial screening biomarker for fish health, particularly in field sampling, gross indices are not as sensitive to endocrine disruption as other endpoints (Pawlowski *et al.*, 2004; Verslycke *et al.*, 2002). Nonetheless, the significant differences in GSI observed between anti-androgen, steroid oestrogen and mixture treatment groups provided an interesting impetus for the histological analysis in experiment two.

The steroid oestrogens and anti-androgenic pharmaceuticals also failed to cause a significant effect on secondary sexual characteristics and no effect was observed in the combined mixture treatment either. Tubercle number and prominence were significantly reduced in the positive control of 10 ng/L EE2, although fatpad index was unaffected. It could be argued that a longer study duration or exposure of younger fish, where these characteristics have not yet developed, could produce a significant effect following exposure to the oestrogen and anti-androgen treatments. However, previous studies have shown that anti-androgenic pharmaceuticals and steroid oestrogens affect these endpoints at higher concentrations than those employed in this experiment. Indeed, 27 ng/L of E2 caused a reduction in both tubercles and fatpad in one study (Miles-Richardson *et al.*, 1999), whilst Salierno *et al.* found similar results to the positive control, with 10 ng/L EE2 causing a reduction in tubercle number and prominence (Salierno and Kane, 2009). In comparison, the concentrations of anti-androgenic pharmaceuticals capable of inducing similar effects are over 1,000 times above predicted environmental concentrations, based on previous studies. Indeed, flutamide caused a reduction in the tubercle number of males at 938 µg/L (Panter *et al.*, 2004), whereas bicalutamide reduced tubercle prominence at a lower concentration of 100 µg/L (Panter *et al.*, 2012). However, cyproterone acetate at doses up to 200 µg/L failed to have any significant impact (Ankley *et al.*, 2010). Taken together, these data suggest that it is unlikely that the secondary sexual characteristics of wild fish exposed to these pharmaceutical anti-androgens would be affected at their environmental concentrations. Nonetheless, cross species comparison for these endpoints should be made with caution as different fish species can respond differently to similar chemicals. Indeed, urogenital papilla and colouration in zebrafish were significantly feminised at

0.5 ng/L EE2, in comparison to 200 ng/L EE2 required to affect colouration in guppies (Larsen *et al.*, 2008; Nielsen and Baatrup, 2006).

As anticipated, vitellogenin induction provided a much more sensitive endpoint for disruption during this experiment, with the steroid oestrogen treatment inducing a statistically significant vitellogenic response in the fathead minnows. However, the anti-androgenic pharmaceuticals failed to induce a statistically significant response alone. In the combined mixture treatment, the median vitellogenin concentration was three times that of fish in the steroid oestrogen treatment alone, but again this was not statistically significant. This showed that the addition of anti-androgens to steroid oestrogens in this exposure did not have a significant effect on vitellogenin induction. In comparison, previous studies have found that vitellogenin induction is less sensitive to exposure to anti-androgens than it is to the steroid oestrogens (Filby *et al.*, 2007b; Ankley *et al.*, 2004; Jensen *et al.*, 2004). Indeed, one study induced around a six fold increase in male fathead minnows, but only when they were exposed to 500 µg/L flutamide (Jensen *et al.*, 2004). Another study using 320 µg/L flutamide caused only a 1.4 fold increase in males, which was not considered to be statistically significant, in comparison to a 345 fold increase following exposure to 10 ng/L EE2 (Filby *et al.*, 2007b). Induction of vitellogenin by anti-androgens is thought to occur through a similar mechanism to that observed in humans, where an AR antagonist can cause an increase in circulating concentrations of exogenous oestrogens. Indeed, in one fish study where vitellogenin was induced by flutamide, a small increase in plasma E2 was also observed (Jensen *et al.*, 2004). In addition, other molecular mechanisms could be involved, such as the increased expression of the oestrogen receptor β (Filby *et al.*, 2007b), which is thought to be the main mediator of vitellogenin induction (Leaños-Castañeda and Van Der Kraak, 2007). There are no other studies of bicalutamide available for comparison with this endpoint. However, given that it is also an AR antagonist and seems to impact fish at similar concentrations to flutamide, it is plausible that concentrations in the µg/L range may be required to produce similar changes in vitellogenin in male fish. In contrast, cyproterone acetate has been shown to reduce vitellogenin observed in female fathead minnows at 200 µg/L, potentially mediated by its anti-gonadotrophic activity and the associated reduction in plasma sex steroids (Ankley *et al.*, 2010).

3.4.3 Experiment two: Intersex prevalence and severity

Experiment two employed an exposure of Japanese medaka during development from embryonic to adult phases. Whilst survival was above 80% in most treatments, mortality was higher in one replicate from both the control and solvent control treatments. The exact cause for this remains unknown, although the affected control tank did experience higher and more frequent temperature fluctuations than the other tanks. High mortality also occurred in the positive control tanks where fish were all found to be phenotypically female. Previous study has also observed high mortality in fish exposed to E2 at concentrations high enough for complete sex reversal, where hypertrophy of the liver and kidneys occurred alongside an accumulation of proteinaceous fluid, which was assumed to be vitellogenin (Herman and Kincaid, 1988).

Interestingly, no female fish were identified in either the control or solvent control treatments of medaka and they were few in number in the rest of the treatments. Whilst previous studies have shown male majorities in control tanks (53-54% male) (Zha and Wang, 2006; Metcalfe *et al.*, 2001), an additional pressure would be required to induce an all-male population. A likely explanation for this unexpected phenomenon is masculinisation of genotypic females by a cortisol induced stress response to temperature fluctuations (Hayashi *et al.*, 2010; Hattori *et al.*, 2007). Such fluctuations could have been caused during the period of embryonic development, where daily renewal of media in petri dishes occurred. Indeed, the fresh media stock was kept at room temperature (~20ºC) and used to renew the media in the petri dishes, which were constantly maintained between 25 and 26ºC. In addition, E2 has been shown to combat this temperature induced masculinisation response (Kitano *et al.*, 2012), which could explain the presence of females within other treatment groups, particularly the positive control. The modification of characteristics of test organisms prior to or in conjunction with exposure to a chemical of interest is well established in endocrine disruption research, particularly when trying to identify modes of action. Indeed, some tests involve co-exposures of female fish, such as fathead minnows and stickleback, to androgens and anti-androgens, to determine whether the anti-androgen can inhibit masculinisation (Knag *et al.*, 2013; Pottinger *et al.*, 2013; Ankley *et al.*, 2010; Jolly *et al.*, 2009). From this perspective, statistical analysis of intersex induction and severity was repeated to include the presence of females as a treatment induced response, where female fish were considered as intersex index 7. However, the results of this analysis did not differ from the original analysis in terms of the differences in intersex incidence and severity between treatment groups.

The intersex condition has been identified in field surveys of multiple fish species in rivers downstream of anthropogenic input, particularly WWTW effluent discharge, globally (reviewed in Bahamonde *et al.*, 2013). The steroid oestrogens are widely considered to be major contributors to intersex in wild fish. In the UK, their presence downstream of WWTW effluent discharge has been associated with the prevalence and severity of this condition (Jobling *et al.*, 2006). Exposure of fish to effluents containing mixtures of steroid oestrogens have been shown to cause intersex and even complete sex reversal, as have individual chemical exposures (examples include: Zhao *et al.*, 2014; Baynes *et al.*, 2012; Lange *et al.*, 2011; Lange *et al.*, 2009; Kidd *et al.*, 2007; Liney *et al.*, 2006; Balch *et al.*, 2004; Nash *et al.*, 2004; Metcalfe *et al.*, 2001; Rodgers-Gray *et al.*, 2001; Papoulias *et al.*, 2000). However, to the author's knowledge, there have been no other studies assessing intersex in fish exposed to a mixture of E1, E2 and EE2.

This study demonstrated that mixtures of steroid oestrogens are capable of inducing an environmentally relevant incidence and severity of intersex at concentrations comparable with those observed in rivers and effluents. A dose dependant response in intersex incidence and severity was achieved by the low and high doses of both the steroid oestrogen and mixture treatments. This is consistent with studies exposing fish to single steroid oestrogens, increasing dilutions of oestrogenic WWTW effluents and the UK river survey conducted by Jobling *et al.* (Liney *et al.*, 2006; Jobling *et al.*, 2006; Metcalfe *et al.*, 2001; Rodgers-Gray *et al.*, 2001). At the low oestrogen doses in this study, which were established as being relevant to high river concentrations, 24% of males were found to be intersex. In studies of wild fish, the incidence of intersex varies between sites and species. In wild roach from UK river stretches classed as being at high risk of intersex induction, 31.25% of the sampled male roach were found to be intersex in terms of the presence of oocytes in their testes. In annother fish survey from the USA 18-22% intersex occured in white suckers (*Catostomus commersoni*) immediately downstream of an oestrogenic WWTW effluent discharge in Colorado (Vajda *et al.*, 2008). It should be considered that up to 100% intersex in males have been observed at some UK river sites, including individuals with ovarian cavities, which could not be assessed in this study (Jobling *et al.*, 2006; Jobling *et al.*, 1998). Intersex severity in gonad sections from fish in the low oestrogen and mixture groups was predominantly low (index 1-2), although higher severity (index 5-6) was also observed. Again, this corresponded well with data from river surveys (Jobling *et al.*, 2006; Jobling *et al.*, 1998).

At the higher doses, there was a greater proportion of fish with high severity intersex than the lower doses. Indeed, at the higher, effluent relevant doses, 80 and 88% of male fish were intersex in the oestrogen and mixture treatments respectively. Additional changes to gross morphology were also observed, including a high incidence of gonadal disorganisation and extensive fibrotic tissue, the prevalence of which was slightly greater in the high mixture treatment. These abnormalities have been observed previously in controlled exposures to steroid oestrogens (Kidd *et al.*, 2007) as well as anti-androgens, including cyproterone acetate and vinclozolin (Kiparissis *et al.*, 2003). They have also been observed in wild caught fish from effluent contaminated areas (Björkblom *et al.*, 2013; Nolan *et al.*, 2001; Jobling *et al.*, 1998). Fibrosis has also been observed in an exposure of fish to anti-androgenic effluent in Australia where no steroid oestrogens were detected (Kumar *et al.*, 2012). It is likely that severely intersex fish from these higher doses were reproductively impaired due to the extent of disorganisation of the gonad and the observation of empty sperm ducts. This is supported by a study of wild roach, where individuals with high intersex severity were found to have a reduced reproductive performance (Harris *et al.*, 2011).

The induction of intersex by a mixture of steroid oestrogens invites the question of whether this was an effect of a single chemical or a combination of all three steroid oestrogens. Additive effects of steroid oestrogens are well documented and have been observed both *in vitro* and *in vivo*, on endpoints including vitellogenin production, fecundity and secondary sexual characteristics in combination with other xenoestrogens (Brian *et al.*, 2007; Brian *et al.*, 2005; Rajapakse *et al.*, 2002; Silva *et al.*, 2002; Thorpe *et al.*, 2001). However, additive effects have not been demonstrated for intersex, although they are plausible. It should be noted that the concentrations of E2 and EE2 in the low oestrogen treatment were below their reported LOECs of 0.75 ng/L EE2 (Zhao *et al.*, 2014) and 10 ng/L E2 for intersex induction (Metcalfe *et al.*, 2001). In comparison, E1 was detected above its LOEC of 10 ng/L (Metcalfe *et al.*, 2001). However, the 10 ng/L E1 exposure of Japanese medaka during early life only induced 5% intersex, whilst 100 ng/L induced 7% intersex in the same study. In comparison, a much greater intersex incidence of 24% of male fish was observed in experiment two in this study. However, without detailed dose response curves from single chemical exposures it is not possible to mathematically prove additive effects for this endpoint in this study. Nonetheless, it remains clear that environmentally relevant concentrations of steroid oestrogens are major drivers of intersex induction.

In comparison, the anti-androgenic pharmaceuticals failed to induce a statistically significant incidence of intersex in either the low or high anti-androgen treatment tanks.

Although there was a small incidence of low severity intersex in the low anti-androgen dose, this did not differ from the solvent control and no intersex was observed in the higher dose. As a result, this was not considered to be an effect of the anti-androgenic pharmaceuticals. Previous studies have suggested that oestrogenic food sources could impact this endpoint (Bahamonde *et al.*, 2013). However, the ZM fish food used in both studies was not found to have oestrogenic activity *in vitro* (Beresford *et al.*, 2011). Given that no intersex was observed in the second solvent control replicate or the aquarium water controls, contamination of these tanks at some stage in the experiment seems to be the most likely cause of intersex induction. Analytical chemistry found no evidence of such contamination, but with water samples taken and analysed on only four out of the 98 days of exposure, contamination still remains a plausible possibility. Intersex has been observed in control medaka from a number of studies from different laboratories (Grim *et al.*, 2007), although this was considered to occur due to external factors and it is unlikely to be a natural occurrence in this species (Bahamonde *et al.*, 2013; Grim *et al.*, 2007).

It is likely that higher concentrations of anti-androgenic pharmaceuticals than those employed in experiment two are required to induce intersex in fish. Indeed, a multigenerational exposure of fathead minnows to bicalutamide, employing concentrations up to 100 µg/L did not observe any abnormal gonad histopathology in males (Panter *et al.*, 2012). In addition, the other pure AR antagonist, flutamide, required concentrations of 202 µg/L in one study and 320 µg/L in another to induce intersex in Japanese medaka. (León *et al.*, 2007; Kang *et al.*, 2006). On the other hand, cyproterone acetate caused intersex induction at lower concentrations of 1 and 10 µg/L (Kiparissis *et al.*, 2003). This could be due to its additional ability to reduce concentrations of circulating androgens through anti-gonadotrophic effect, alongside its AR antagonism. Indeed, more pronounced effects of cyproterone acetate than flutamide have also been observed in mammalian models and in human trials for prostate cancer treatment at similar doses (Barradell and Faulds, 1994). However, the predictive hydrological modelling suggests that these concentrations greatly exceed those present in the aquatic environment. Nonetheless, cyproterone acetate has been shown to reduce both the testicular synthesis and plasma concentrations of testosterone and 11-ketotestosterone in mummichog at the concentrations employed in both experiments one and two (Sharpe *et al.*, 2004). These endpoints were not assessed in this study and if plasma sex steroids were altered they did not translate into any significant impacts on other visible endpoints, such as secondary sexual characteristics.

Different chemicals with similar mechanisms of action, such as AR antagonists and steroidogenesis inhibitors, have been shown to act additively *in vivo* (Christiansen *et al.*, 2009). Statistical modelling of the occurrence of steroid oestrogens and anti-androgenic activity at capture sites with observed intersex and vitellogenin induction in wild fish has suggested that these two sets of chemicals could interact to cause sexual disruption (Jobling *et al.*, 2009). Interestingly there was a 9-10% increase in intersex incidence in the mixture treatments at both doses in comparison to steroid oestrogen treatments alone. However, statistical analysis found that this was not a statistically significantly increase. At present, there is only one other study that assessed the effects of mixtures of anti-androgens and steroid oestrogens in fish, for which only preliminary data has been published (Lange *et al.*, 2012a). This reported a significant increase in the incidence of ovarian cavities in juvenile roach following exposure to a combination of anti-androgens identified in WWTW effluents and steroid oestrogens. Unfortunately the longitudinal nature of the histological sectioning in medaka was not effective in accurately identifying ovarian cavities in males and so the Lange study could not be directly compared by this study. Consequently, a multi-causal aetiology behind intersex induction remains worthy of future study. This could perhaps employ a mixtures exposure examining both ovarian cavities and germ cells to determine definitively whether anti-androgens and oestrogens interact in an additive or synergistic fashion in fish.

3.5 Conclusions

This study used hydrological modelling to predict that bicalutamide and cyproterone acetate are likely to be contaminants of the aquatic environment which are widespread across river catchments in England and Wales. In the majority of cases, their concentrations are likely to occur below 10 ng/L but at some "hot spots," concentrations are likely to be higher, in some cases exceeding 100 ng/L for bicalutamide. When fish were exposed to high environmental concentrations of these pharmaceuticals alone and in combination with steroid oestrogens, no significant effects were observed on a set of endpoints indicative of sexual disruption. It is likely that higher concentrations in µg/L are required to induce significant effects, which are potentially up to 1,000 times greater than those identified in the environment. However, given the evidence for additive effects of anti-androgenic chemicals in whole organisms, these environmental contaminants should be considered as part of the wider issue of anti-androgenic activity in the environment. Critically, this study

demonstrates that a mixture of steroid oestrogens, at concentrations present in the aquatic environment, can induce intersex at a rate comparable with that observed in UK and European rivers. Additional exposures to anti-androgenic pharmaceuticals known to co-occur with these oestrogens did not exacerbate the incidence or severity of intersex. Taken together, these data support the role of steroid oestrogens as major contributors to intersex in wild fish.

CHAPTER 4: IDENTIFICATION OF ENVIRONMENTAL ANTI-ANDROGENS THROUGH EFFECT DIRECTED ANALYSIS

4.1 Introduction

Anti-androgenic activity has been detected at a high frequency in wastewater treatment works (WWTWs) effluents, surface waters and sediments. In many cases, the flutamide equivalent concentrations (FluEQ) that these samples produce *in vitro* exceed concentrations of flutamide known to have adverse effects on fish in laboratory studies (Grover *et al.*, 2011; Zhao *et al.*, 2011; Johnson *et al.*, 2007b; Urbatzka *et al.*, 2007). However, the causes of this activity remain to a large extent unidentified and targeted analysis of the concentrations of known anti-androgenic chemicals, such as bisphenol A (BPA), alkylphenols and pesticides, have failed to fully explain the detected activity (Kinani *et al.*, 2010). Consequently, the identification of anti-androgenic chemicals responsible for the observed anti-androgenic activity in complex environmental samples remains a key challenge, to which more in depth and broad ranging approaches need to be applied.

4.1.1 Identifying the chemical causation of biological effects in complex environmental samples

Complex environmental samples, such as WWTW effluents, can contain many thousands of chemicals (Jobling and Tyler, 2003). Due to the limitations of analytical chemistry, such as the need for multiple methods and the lack of available methodologies for some chemicals, it is not possible to identify every single chemical, particularly without prior knowledge of its likely contents. Even if this were possible, biological and chemical assessment of potentially thousands of chemicals would be vastly expensive and time consuming (Hecker and Hollert, 2009). Consequently, two different approaches have been developed to direct both chemical and biological analysis to identify the chemicals causing a biological effect in an active environmental sample. These are the Toxicity Identification Evaluation (TIE) and Effect Directed Analysis (EDA) (Burgess *et al.*, 2013), both of which use tiered approaches employing a combination of analytical chemistry and bioassays, with the intention of including the maximum possible number of chemicals in their assessment.

4.1.1.1 Toxicity Identification Evaluation (TIE)

TIE was developed by the US EPA in response to the Clean Water Act to identify toxic chemicals in complex mixtures based on a three tiered approach of characterisation, identification and confirmation of toxicity. It is generally associated with whole organism

bioassays, mainly using invertebrates such as *Daphnia magna*, with endpoints such as reproduction and survival. In a TIE, when an environmental sample is found to be toxic in these bioassays, phase I analysis is completed to characterise the responsible class of chemicals through their physiochemical properties (Burgess *et al.*, 2013; Ankley *et al.*, 2011). Critically, since TIE involves detecting toxicity in a whole sample, not a solvent extract, the causal chemicals are characterised through manipulations of an environmental sample itself to preserve the link between the sample and the observed toxicity (Ankley *et al.*, 2011). Examples of these include pH adjustment and filtration, which are then combined with whole organism toxicity tests. For example, if the toxic effect is not observed following filtration, it is likely to be caused by particulate associated toxicants. After chemical characterisation, phase II identification can be directed to identify the causal chemicals within the class identified in phase I, through analytical chemistry such as gas chromatography-mass spectrometry (GC-MS). Phase III confirmation then involves producing independent lines of evidence to support findings from phases I and II. This can involve another whole organism test with exposure to the suspected toxicants to determine whether similar toxic effects occur in comparison to the original sample (Burgess *et al.*, 2013).

4.1.1.2 Effect Directed Analysis (EDA)

The key difference between EDA and TIE is that analysis is not directed by whole organism tests for toxicity but through *in vitro* screens for biological activity. Consequently, unlike the TIE process, EDA uses extraction and fractionation procedures to concentrate the sample for assessment in a solvent extract form. This also means that biological samples where chemical concentrations are low, such as blood or tissue, can also be assessed through EDA. A basic EDA starts with a sample where high biological activity has been detected in an appropriate bioassay. Broad spectrum solid phase extraction (SPE) methods are then used to maximise the capture of chemicals within the active sample. This is followed by fractionation of the extract by high performance liquid chromatography (HPLC) to separate chemicals based on their polarity. The fractions are then individually screened in a bioassay and can be further fractionated and rescreened if necessary. Chemical analysis, again such as GC-MS, can then be used to identify specific chemicals in the active fractions. Like in TIE, confirmation is then required, which normally involves a bioassay of the suspected chemical standards to determine their contribution to total activity of the active sample (Burgess *et al.*, 2013; Hecker and Hollert, 2009). Since there is no whole organism test

included within the EDA, this can be added into a hazard confirmation step with exposures to chemicals standards.

4.1.2 Effect directed analysis and endocrine disrupting chemicals

EDA provides a useful assessment technique for assessing endocrine disrupting chemicals (EDCs) in complex samples since it can be directed by *in vitro* bioassays for endocrine disrupting activity. In addition, since samples can be concentrated through SPE, the accuracy of analytical chemistry is vastly improved. This is particularly important for high potency chemicals, such as 17α-ethinylestradiol (EE2), which has a lowest observed effect concentration (LOEC) that is below the limits of detection of some analytical techniques. The steroid oestrogens were first identified in WWTW effluent as the major cause of oestrogenic activity through a process described as a *"TIE approach modified for our specific needs"* (Desbrow *et al.*, 1998). In fact, this is more akin to an EDA through its use of extraction, fractionation and direction by an *in vitro* bioassay, the yeast oestrogen screen (YES). The process is shown below in Figure 4.1, in which an oestrogenic WWTW effluent was purged with nitrogen to remove volatiles and filtered to remove particulates and assessed with the YES bioassay. This found that the activity remained in the aqueous phase of the effluent. The effluent was then concentrated in a broad spectrum SPE using a variety of elution solvents and solvent concentrations to extract a wide polarity range of contaminants before the extracts were fractionated with HPLC. YES was then used to direct analysis towards active fractions, which underwent reconcentration and further fractionation before being analysed with GC-MS, which eventually detected the steroid oestrogens oestrone (E1), 17β-oestradiol (E2) and EE2 in active fractions.

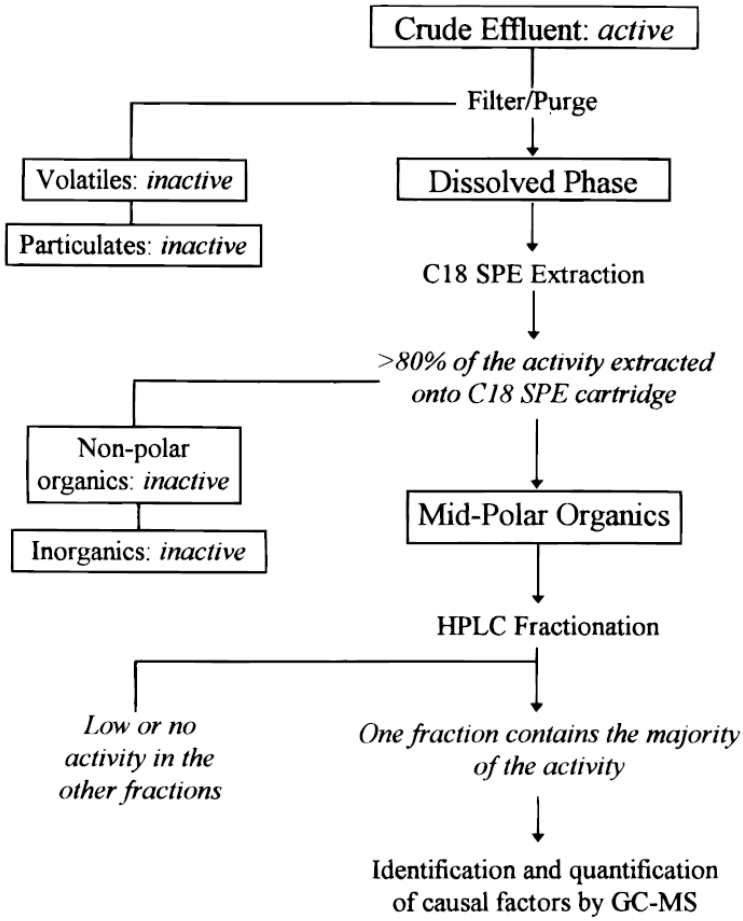


Figure 4.1 *The process employed to identify the causes of oestrogenic activity in WWTW effluent which was directed towards the steroid oestrogens* (from Desbrow *et al.*, 1998).

Further EDA of environmental samples have also identified steroid oestrogens as major or even dominant contributors to oestrogenic activity. Indeed, a basic fractionation procedure completed on effluent, river and lake water samples in the US found that E2 and EE2 accounted for 88-99.5% of total sample activity (Snyder *et al.*, 2001). However, other xenoestrogens have also been detected in active fractions in other studies. In samples from UK estuaries, nonylphenol was found to be a major contributor to the activity alongside E2. In addition, the phthalate ester di (2-ethylhexyl) phthalate (DEHP) was also thought to significantly contribute to activity but the link remained tentative since DEHP is a common laboratory contaminant (Thomas *et al.*, 2001). Another study of sediments from the River Elbe, Germany, found that mixtures of xenoestrogens may contribute to activity to an extent which equals the steroid

oestrogens (Schmitt *et al.*, 2012). However, it should be considered that environmental samples are unique in their chemical constituents based on their surrounding geography, local contaminant sources and the time of sampling. As a result, the dominant chemicals may differ between sites. Indeed, a study of sediments from the upper Danube River, Germany, found that only 2-6% of oestrogenic activity could be linked to the steroid oestrogens, with the rest of the activity coming from unknown contaminants (Grund *et al.*, 2011). However, in this case the limits of detection for E2 and EE2 were higher than those of E1. It is therefore possible that these chemicals were present and making significant contributions to activity but were not detected.

4.1.3 Effect directed analysis and anti-androgens

EDA has also been applied to assess anti-androgenic activity in environmental samples through androgen receptor (AR) transfected bioassays such as the yeast androgen screen (YAS). Here, the assessment of activity is based on the ability of a sample extract to reduce an androgenic response mediated by a background concentration of 5α-dihydrotestosterone (DHT) in the yeast cell line. The assay has been coupled with targeted chemical analysis, assessing concentrations of a set of known anti-androgens within active fractions from environmental samples. This identified anti-androgens such as BPA, alkylphenols (monylphenol [NP] and octylphenol [OP]) and pesticides (vinclozolin, endosulfan, dichlorodiphenyltrichloroethane [DDT], *p'p*-dichlorodiphenyldichloroethylene [*p'p*-DDE] and iprodione) in river sediments from France and Italy (Kinani *et al.*, 2010; Urbatzka *et al.*, 2007). However, these were found to make only minor contributions to the total activity, suggesting that other contaminants have a significant role to play (Kinani *et al.*, 2010). Consequently, broad ranging analytical techniques and chemical identification software have been used in EDAs to tentatively identify more unknown chemicals. For example, chemical analysis of active fractions from produced water discharges from offshore oil production platforms used high resolution time of flight mass spectrometry, which detected anti-androgenic polyaromatic hydrocarbons (PAHs) and naphthenic acids (Thomas *et al.*, 2009). However, a recent *in vivo* exposure to commercial mixtures of naphthenic acids found no evidence of anti-androgenic effects in fish (Knag *et al.*, 2013). PAHs have also been detected in active fractions of river sediments alongside alkylphenols and the phthalate ester, DBP (Weiss *et al.*, 2009). This last study also highlighted the importance of EDA in assessing endocrine activity in samples, where androgenic activity was found in some fractions, which had been concealed by the anti-androgenic activity in the total extract.

One advantage of EDA is its ability to assess biological samples, such as blood or tissue, through the use of sample concentration. The bile of fish exposed to WWTW effluent has been successfully used for assessments of EDCs since it bioaccumulates contaminants to concentrations greater than those that exist in the effluent itself (Hill *et al.*, 2010). Indeed, oestrogenic activity has been identified through EDA in fractions of fish bile and shown to contain steroid oestrogens, alkylphenols and BPA (Pettersson *et al.*, 2006; Gibson *et al.*, 2005). A similar method has also been completed for identifying anti-androgens in fish exposed to anti-androgenic effluent. This identified a broad range of chemicals including PAHs, chlorinated PAHs, alkylphenols, BPA and isomers of resin acids, as well as chemicals originating from personal care products, such as biocides (triclosan and chlorophene) and oxybenzone, which is used in sunscreen filters (Rostkowski *et al.*, 2011; Hill *et al.*, 2010). Interestingly, the biocides triclosan and chlorophene were shown to be the most potent anti-androgens, accounting for 51% of the total activity. However, even with the addition of the other identified chemicals only around 60% of the total activity could be accounted for.

4.1.4 Aims and objectives

The objective of this study was to use EDA in a broad approach to identify anti-androgenic contaminants in WWTW effluent and river samples from the UK. This provides an alternative method for identifying anti-androgenic environmental contaminants to complement the targeted approach of assessing the role of pharmaceuticals described in Chapters 2 and 3. This chapter aimed to identify other anti-androgenic chemicals, quantify their individual potency and determine their contribution to anti-androgenic activity. A similar process is also currently underway using WWTW effluents from five South Australian WWTWs.

4.2 Materials and methods

4.2.1 Effect directed analysis overview

A schematic of the EDA process is shown in Figure 4.2. In this study, two sets of samples were collected from WWTW final effluents and extracted by SPE. One set underwent HPLC fractionation and the resultant fractions were analysed *in vitro*, with the yeast androgen screen (YAS), to identify (anti-) androgenic activity. The intention was to use the YAS results to direct analysis towards chemicals in active fractions to identify the anti-androgenic constituents of the effluent. The fractions also underwent a

broad scan by GC-MS, where their constituent chemicals were tentatively identified through comparison with a mass spectral library. Literature searches were then used to label chemicals identified by GC-MS as those with known anti-androgenic activity. These were then tested in the yeast screen to assess their potential contribution to anti-androgenic activity seen in the whole effluent extracts, which also underwent screening with the YAS. At the same time, some river samples from upstream of WWTWs were also collected, extracted and fractionated to assess their chemical constituents. SPE, HPLC fractionation and GC-MS were all completed at Severn Trent Services, Reading, whilst yeast screening was completed at Brunel University, London. Additional background material on SPE, HPLC and yeast screening is provided in Appendix 3.

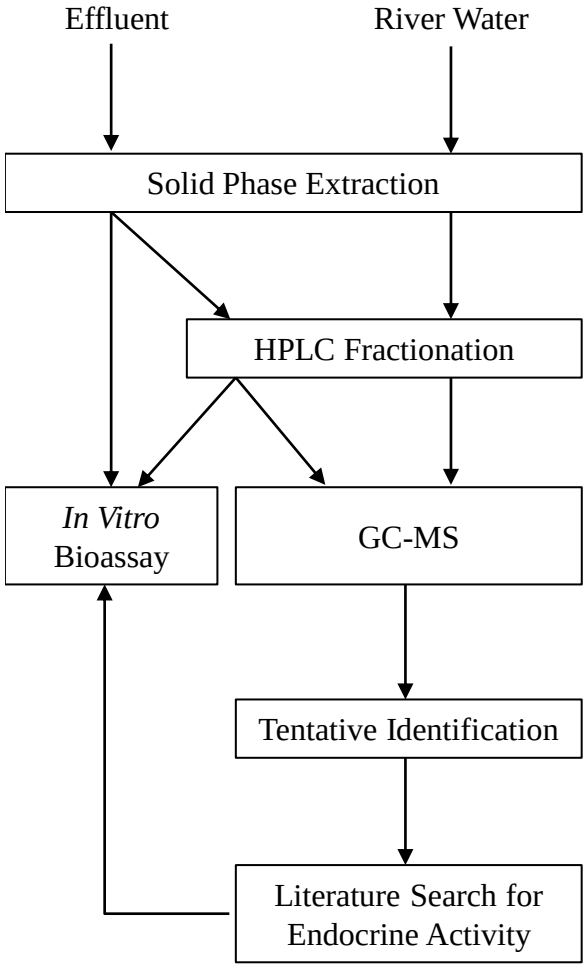


Figure 4.2 *The Effect Directed Analysis process used in this study to identify anti-androgenic chemicals in UK effluent and river water samples.*

4.2.2 Sites and sample collection

Effluent samples from the WWTWs, UK1 and UK3 (Severn-Trent catchment, UK Figure 4.3), were analysed in this study. These had been previously involved in Chapter 2's predictive modelling approach. UK1 is a relatively rural WWTW located near the town of Alfreton, Derbyshire. It served an estimate of 53,695 people with an average domestic flow of 8.17 ML/day based on 2009 data and discharges into Alfreton Brook, which in turn discharges into the River Amber. The larger WWTW, UK3, is located in Longbridge near the town of Warwick, which served an estimated population of 117,041 with an average domestic flow of 11.41 ML/day, which discharges into the larger River Avon. Both employed similar treatment processes which included primary settled sewage, secondary biological trickling filter and a tertiary sand filter. These WWTWs were chosen since preliminary investigation had identified anti-androgenic activity in both final effluents with an average of 407 and 325 µg/L FluEQ for UK1 and UK3 respectively (Baynes *et al.*, 2014 personal communication).

In February 2009, grab samples of final effluent from each WWTW were collected using a stainless steel sampling can and transferred into 2.5 L amber glass Winchesters containing copper (II) nitrate (0.5 g) and hydrochloric acid (6 mL) as a fixative. These were then stored at 4°C to inhibit possible bacterial degradation of chemicals. Two replicate samples were collected from each WWTW. One set underwent extraction, HPLC fractionation and in vitro screening, whilst the other was extracted and screened to assess the activity in the whole effluent sample, to compare with the activity of the fractions and the chemicals identified within them. River water samples were also collected at this time from upstream of the UK3 discharge point, since the Avon provides a good example of a rural, agriculturally impacted river site. To compare with this, water samples were also collected from the urban and industrialised River Erewash. These samples were collected and preserved using the same method as the effluent samples.

Figure 4.3 *Images of the WWTWs and the waterways they discharge to* (sourced from Google Maps).

4.2.3 Solid phase extraction (SPE)

In this EDA approach, SPE was used to capture a broad range of the chemicals present in the effluent and river samples and to concentrate them to improve the accuracy and detection frequency of GC-MS. The effluent and river samples underwent reverse phase SPE on styrene divinyl benzene cartridges (Isolute ENV+), which can capture chemicals with a broad range of polarities. Cartridges were attached to an extraction manifold and conditioned with 5 mL of ethyl acetate followed by 5 mL of methanol and 5 mL of water in sequence. Each 2.5 L sample was then loaded onto the cartridge under vacuum before being thoroughly dried. The samples were then eluted into collection vessels with 5 mL of dichloromethane and 5 mL of methanol to maximise the number of chemicals eluted. The 10 mL extracts were then concentrated by

Turbovap LV automated evaporation system to 1mL before further concentrating them with nitrogen blow down equipment to 100 µL in a 1 mL autosampler vial.

4.2.4 Fractionation by high performance liquid chromatography (HPLC)

This study used reverse phase HPLC fractionation to separate chemicals from the SPE extracted samples into fractions based on their polarity using an elutrophic series in the polar mobile phase and a non-polar stationary phase. This employed a HPLC system (Hewlett Packard 1050 HPLC) with a quaternary pump, column heater and UV detector, which was connected to a Dynamax fraction collector (model FC-4). The system was maintained at ambient temperature with a C18 based column (Phenomenex Gemini 5µm C18 110A, 150mm x 4.6 x 5µm. Part No. 00F-4435-E0). The sample was injected at a volume of 95 µL with the mobile phase flowing at 1 mL/minute, starting as 5% methanol in distilled water and changed linearly to 100% methanol after 20 minutes before being maintained at 100% after 27 minutes. Fractions of 1 mL were collected every minute for 30 minutes, producing 30 fractions for *in vitro* assessment of anti-androgenic activity and GC-MS analysis.

4.2.5 *In vitro* identification of anti-androgenic effluent fractions

Effluent fractions were tested for androgenic and anti-androgenic activity using the *in vitro* yeast androgen screen to direct analysis towards anti-androgenic chemicals. Fractions were screened based on the protocol detailed in (Sohoni and Sumpter, 1998) to detect androgenic and anti-androgenic activity in YAS. This was completed in a type II laminar flow cabinet, where 100 µL of a fraction was serially diluted at 12 concentrations in 100 µL methanol in 96 well microtitre plates. Duplicate 10 µL aliquots of each concentration were then transferred to a second set of plates. These plates also contained a blank, solvent control (methanol) and flutamide as a positive control, which were run alongside the fractions in duplicate. Like the fractions, flutamide was also serial diluted across the wells at concentrations from 50 to 0.02 nM to produce a sigmoidal response curve. These were allowed to evaporate to dryness before 200 µL of media seeded with $4x10^7$ yeast cells, CPRG and a background concentration of 2 nM DHT was added to each well. The plates were then placed in a mictotitre plate shaker for 2 min before being incubated at 32^oC. After 24 hours the plates were shaken again to disperse the growing cells and were then returned to the incubator. After 72 hours the plates were analysed using a Spectramax Plate Reader (Molecular Devices,

USA), measuring absorbance at wavelengths of 540 nm for colour change and 620 nm for cell turbidity. Each well was corrected for turbidity based on its 540 nm absorbance minus (620 nm absorbance – solvent control 620 nm absorbance). In these screens, the turbidity reading was essential since toxicity of a chemical can produce a similar result to anti-androgenic activity by reducing the number of cells available to produce a response. Consequently, if excessive toxicity was identified, the sample was discounted since it could not be differentiated from anti-androgenic activity. The same method was used to assess whole effluent samples.

4.2.6 Chemical analysis by gas chromatography-gas spectrometry (GC/MS)

Prior to GC-MS, each sample fraction required a solvent exchange. Each fraction was added to 200 mL of laboratory blank water, pH adjusted to pH 6.5-7.5. Deuterated internal standards, including d_6-benzene, d_5-chlorobenzene, d_{10}-xylene, d_5-phenol, d_8-naphthalene, d_{34}-hexadecane and d_{10}-phenanthrene, were added to each sample based on Table 4.1. Each fraction was extracted to 100 mL dichloromethane by liquid-liquid extraction in a 1 L separating funnel. The pH of the fractions was then readjusted to pH 10 and re-extracted with another 100mL of dichloromethane. The two extracts for each fraction were then combined and the residual water was frozen out overnight in a freezer. The solvent extracts were then concentrated by a Turbovap LV automated evaporation system to approximately 0.5 mL before further concentrating them with nitrogen blow down equipment to 100 µL in a 1 mL autosampler vial. A blank sample was also extracted based on this method for GC-MS and comparison with the fractions.

Table 4.1 *Internal standards added to fractions prior to GC-MS analysis, including their concentrations in the fractions, retention time and mass/charge ratio.*

Internal Standards	Concentration (mg/mL)	Retention Time	m/z
d_6 benzene	2	4.51	84
d_5 chlorobenzene	2	9.4	117
d_{10} p xylene	1	10.07	116
d_5 phenol	8	12.6	99
d_8 naphthalene	1	16.9	136
d_{21} BHT	8	22.4	222,240
d_{34} hexadecane	1	23.8	66,98
d_{10} phenanthrene	2	26.32	188
d_{62} squalene	8	35.9	62, 82

GC-MS couples the separation of chemicals by gas chromatography with their characterisation by mass spectrometry. In gas chromatography, a sample is injected into the instrument, where it is heated on a gradient up to around 300^{o}C and transported through a separation column (stationary phase) with up to 60 m length by an inert gaseous mobile phase, such as helium. As the mobile phase passes through the column, chemicals are separated based on their volatility and their interaction with the stationary phase, where those with high volatility move the fastest as they take less time to evaporate. The chemicals then pass from the GC to the mass spectrometer, which is separated into three main sections: the ion source, mass filter and detector. When they enter the ion source, the chemicals are ionised, fragmented and then focussed into an accelerated ion beam into the mass filter, which separates the ions based on their mass-charge (m/z) ratio. Electromagnets within the filter can be set to control which ions will enter the detector, which detects the mixture of molecular and fragment ions that create a compound's unique spectra. Unfragmented molecular ions can be identified based on their exact masses.

In this study, effluent fractions, river water extracts and the blank underwent GC (Agilent 6890) with a fused silica capillary column (Aglient J&W DB1 60m x 0.32mm x 0.25µm film thickness), where 1 µL of each extract or fraction was injected by split-split headed injector. The temperature program began at 35^{o}C for 4 minutes followed by an increase to 300^{o}C at a rate of 8^{o}C/min, holding at 300^{o}C for 10 minutes. The mobile phase was helium with a flow rate of 1.5 mL/min. This was coupled with a quadruple mass selective MS detector (Aglient 5973), equipped with an electron ionisation source at a temperature of 250^{o}C with an electron energy of 70 eV. Acquisition was achieved

by a general scan survey mode which carried out scanning over a range of 500 to 35 u (unified mass unit) at a scan speed of 0.5 seconds per decade to give a scan cycle time of 0.97 seconds. This broad scanning process aimed to maximise the range of chemicals detected in each fraction.

4.2.7 Tentative chemical identification and semi-quantification

Mass spectra were interpreted by manual searching of the Wiley Register™ of Mass Spectral Data and by mass spectral interpretation from first principals. The chemicals from all effluent, river and blank fractions were either tentatively identified or labelled as unknowns. Concentrations of chemicals of interest were semi-quantified by comparing the peak area of the chemical with those of the internal standards detailed above and added prior to liquid-liquid extraction. The blank sample was used to identify contaminants of the EDA process originating from sample preparation, fraction collection and GC-MS.

4.2.8 Identification of anti-androgens

Literature searching was completed between 2011-12 to identify known anti-androgens from those tentatively identified in effluent, river and blank fractions using the Google search engine alongside the pubmed database. These searches started with active anti-androgenic fractions from WWTW effluents in a targeted approach based on *in vitro* screening (describe below) but were later expanded to all fractions from all samples. The search terms employed included “antiandrogen”, “anti-androgen”, “androgen”, “androgen receptor” and “endocrine disruption.”

4.2.9 Screening of known and suspected anti-androgens in active fractions

To confirm anti-androgenic activity, standards of compounds previously reported as anti-androgenic in the literature or determined to be of interest for screening were analysed in YAS. These were purchased from Sigma-Aldrich, UK and made up in ethanol. These were first tested in the anti-androgen screen with a DHT raised background at six concentrations in a serial dilution (0.5 mM-1.56 µM) as single replicates to make a preliminary assessment of their activity. Chemicals reducing the raised background were retested at 12 concentrations in duplicate and at

concentrations below toxicity, which were specific to each chemical. These assays included a flutamide positive control and an ethanol solvent control. FluEQs were produced based on the following calculation:

$$FluEQ = \frac{x\ (flutamide)\ required\ to\ produce\ y}{x\ (sample)\ required\ to\ produce\ y}$$

Where *x* is the molar concentration and *y* is the corrected 540 nm absorbance. The linear portion of the flutamide curve (the working range) was used to generate comparative data. The concentration of a sample required to produce a given absorbance within the working range was used as the basis to determine the respective concentration of flutamide required to produce the same absorbance. This was calculated using logarithmic regression, as shown in Figure 4.4. This was determined at multiple points within the working range to provide an average FluEQ for each chemical. For example, in the case of pyrene below, three points were within the working range and used to produce the FluEQ.

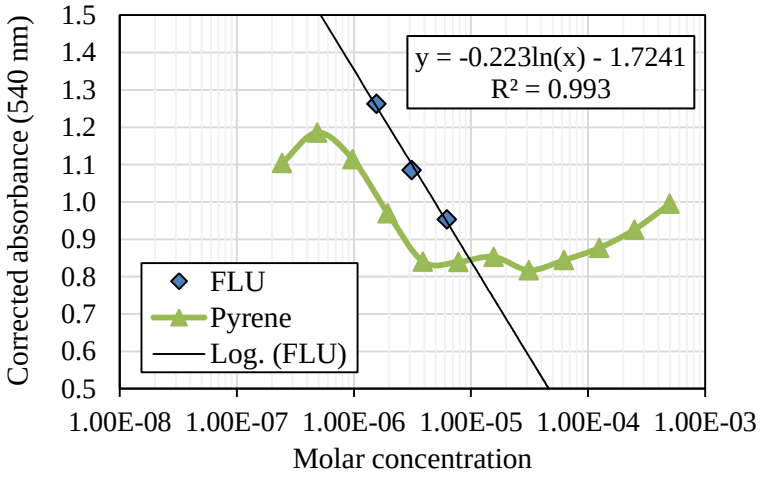


Figure 4.4 *Determining the flutamide equivalents of pyrene using logarithmic regression.*

4.3 Results

4.3.1 Anti-androgenic activity in WWTW effluent fractions

Whole effluent extracts from UK1 and UK3 were confirmed as anti-androgenic and found to contain 242 and 325 µg/L FluEQ. To direct chemical analysis towards anti-

androgens, replicate effluent extracts underwent HPLC to produce 30 fractions of each effluent sample. These underwent screening in the yeast androgen screen with the background raised by DHT to detect anti-androgenic activity. The results of this analysis is summarised below in Figure 4.5 and the raw data is available in Appendix three. Full sigmoidal dose response curves were not achieved by the concentrations tested in the YAS, although the partial curves were indicative of both anti-androgenic and androgenic activity co-occurring in both effluents. Indeed, the corrected absorbance at 540 nm of the serially diluted fractions diverged from the controls at the higher concentrations in the first five wells, where both increases and decreases in absorbance occurred. This allowed active fractions to be tentatively identified, although accurate quantification and comparison with the activity of the whole effluent extracts was not possible. Some confounding factors were identified during this process, producing false positive results for some fractions, which were discounted accordingly. In particular, the edge effect was observed, which was characterised by a reduction in the corrected 540 nm absorption in both the high and low concentration wells on the edges of the plates, producing a shallow U shaped curve. As a result, reductions in 540 nm absorbance in the high concentration wells in some of these cases may have, at least in part, been an artefact from the assay procedure and therefore could not be confirmed as anti-androgenic activity.

A similar number of anti-androgenic fractions were identified in the individual effluents, nine and six for UK1 and UK3, respectively. Five fractions were also shown to have anti-androgenic activity in both effluents. As part of an EDA procedure, active fractions could have been further fractionated to narrow down the group of chemicals causing the activity. However, in this case the number of active fractions would have made further fractionation a time consuming process, with 30 new fractions produced from each active fraction and requiring *in vitro* testing. Consequently, broad scan GC-MS was completed on the fractions to tentatively identify their constituent chemicals, followed by literature searching to identify known anti-androgens. These then underwent confirmation of their activity in the YAS to determine which chemicals, if any, could potentially explain a proportion of the total activity that had been previously observed.

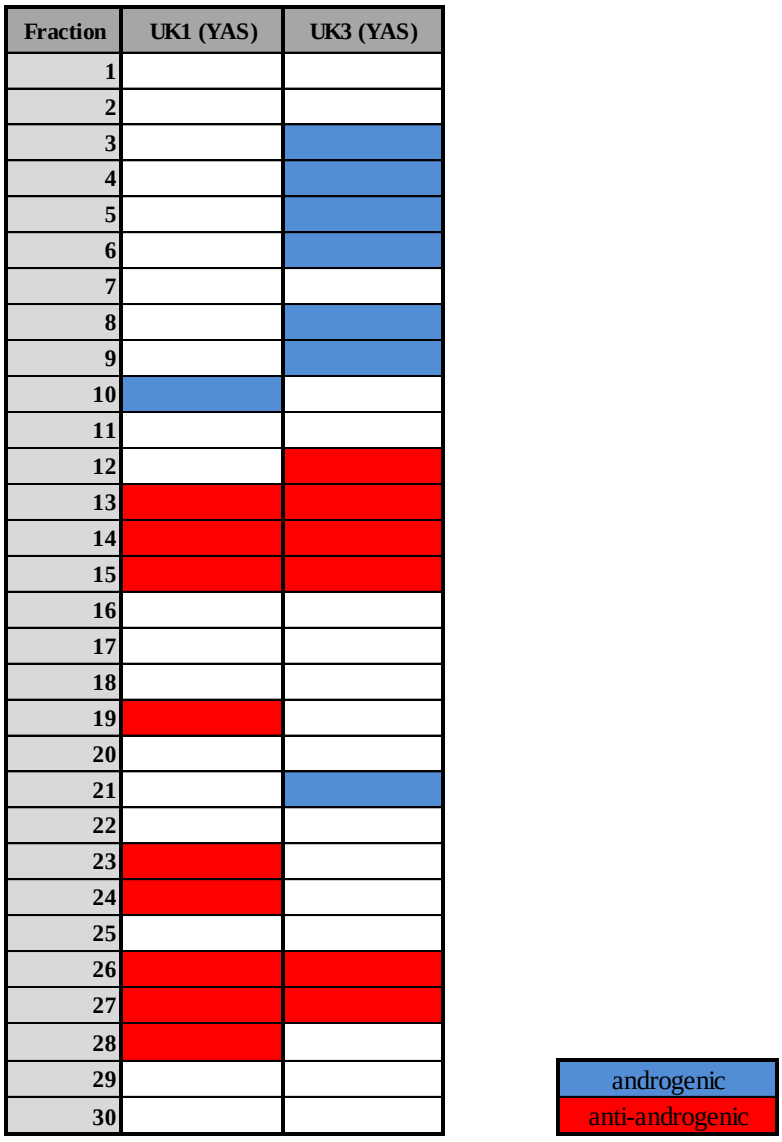


Figure 4.5 *A comparison of (anti-) androgenic activity in effluent fractions from the WWTWs UK1 and UK3, as indicated by their dose response curves in the YAS.*

4.3.2 Identifying and assessing anti-androgens in environmental samples

The chemicals in fractions from effluents, river samples and the blank were tentatively identified from their mass spectra following GC-MS and are summarised in Table 4.2. The highest number of chemicals were detected in the effluents, where 1,063 and 982 were detected in in UK1 and UK3, respectively. As expected the number of chemicals in the river samples was lower, with 268 from the Avon and 243 from the Erewash. 136 possible contaminants were observed in the blank. Although large numbers of chemicals were detected, the vast majority could not be identified (84% at UK1 and 75% at UK3) by this analytical method and the mass spectral library.

Literature searching of the known chemicals found them to have multiple origins. Effluents contained a number of chemicals clearly linked to domestic, anthropogenic use, including fatty acids, fragrances/flavourings, personal care products and pharmaceuticals. Pesticides and a herbicide were also found in effluents and the more rural River Avon. Other chemicals identified included plasticisers, flame retardants and industrial chemicals used in manufacturing as well as some natural products. Interestingly, the sample from the urban River Erewash was found to contain a number of PAHs (polyaromatic hydrocarbons), which were not present in the River Avon.

Table 4.2 *The number of chemicals in detected effluent, river and blank samples. This includes the number of unknown and tentatively identified known chemicals, as well as their uses or origins where they could be determined.*

	Alfreton	Longbridge	Avon	Erewash	Blank
TOTAL	1,064	801	194	241	135
Unknown chemicals	906	558	127	148	85
Known chemicals	158	243	67	93	50
Anti-Oxidant	0	1	1	2	0
Fatty Acid	20	27	7	6	0
Fatty Alcohol	0	0	0	1	0
Flame Retardant	5	6	2	3	0
Fragrance/Flavouring	16	25	6	9	5
Herbicide	0	1	1	0	0
Industrial Byproduct	1	0	0	0	0
Industrial Intermediate	2	4	1	1	0
Organic Synthesis	2	0	2	0	0
Polyaromatic Hydrocarbons	1	0	0	11	0
Personal Care Products	11	17	3	3	4
Pesticide	1	3	0	0	0
Pharmaceutical	5	8	0	0	0
Phytochemical	2	3	0	0	0
Plasticiser	1	3	1	0	0
Precursor Compound	7	9	7	4	2
Solvent	3	3	1	4	1
Surfactant	2	2	0	1	1

Literature searches of compounds by name identified 16 as known anti-androgens. These originated from a range of different classes, including fatty acids, flame retardants, PAHs, pharmaceuticals and personal care products, pesticides and plasticisers. Interestingly, four 5α-reductase inhibitors were also identified alongside the

AR antagonists and it was reported that some fatty acid compounds, such as 9-octadecanoic acid methyl ester, could act through both mechanisms (Selvin *et al.*, 2009). A similar number of known anti-androgens were identified in the effluent samples, with nine found in UK1, 12 in UK3 and seven found in both effluents. In comparison, river samples contained fewer known anti-androgenic chemicals, with three found in the Avon sample and five in the Erewash, of which only one corresponded between the two river samples. Some of these were also detected in the WWTW effluents, such as hexadecanoic acid methyl ester. These chemicals are summarised in Table 4.3 below.

Table 4.3 *Chemicals identified in UK effluent fractions with suspected anti-androgenic activity based on literature sources which were advanced to in vitro testing.*

Compound	Other Names	CAS Number	Descriptor	Origin	Sample
9-Octadecenoic acid methyl ester	Methyl oleate	112-62-9	AR Antagonist/5α reductase inhibitor[1]	Fatty Acid	UK1, UK3
9,12-Octadecadienoic methyl ester	Linolenic acid methyl ester	2566-97-4	Weak 5α Reductase Inhibitor[2]	Fatty Acid	UK1, UK3
Decanoic acid		334-48-5	5α Reductase Inhibitor[2]	Fatty Acid	Avon
Dodecanoic acid	Lauric Acid	143-07-7	5α Reductase Inhibitor[2]	Fatty Acid	UK1, UK3
Hexadecanoic acid methyl ester	Methyl Palmitate	112-39-0	AR Antagonist/5α Reductase Inhibitor[1]	Fatty Acid	UK1, UK3, Avon, Erewash
Pentadecanoic acid		1002-84-2	5α Reductase Inhibitor[2]	Fatty Acid	UK3
Tetradecanoic acid	Myristic acid	544-63-8	5α Reductase Inhibitor[2]	Fatty Acid	Avon
Tri (butoxyethyl) phosphate		78-51-3	AR Antagonist[3]	Flame Retardant	UK3
Tri-n-butyl phosphate		126-73-8	AR Antagonist[3]	Flame Retardant	UK1, UK3
Triphenyl phosphate		115-86-6	AR Antagonist[3,4]	Flame Retardant	UK1
Trischloroethyl phosphate	Tris(2-chloroethyl) phosphate	115-96-8	AR Antagonist[3]	Flame Retardant	UK3
Pyrene		129-00-0	AR Antagonist[5]	PAH	UK1
11H-benzo[b]fluorene		243-17-4	AR Antagonist[6]	PAH	Erewash

Table 4.3 *continued….*

Benzo[a]pyrene		50-32-8	AR Antagonist[5]	PAH	Erewash
Chrysene		218-01-9	AR Antagonist[5]	PAH	Erewash
Fluoranthene		206-44-0	AR Antagonist[5]	PAH	Erewash
4-Chloro-2-(phenylmethyl) phenol	Chlorophene	120-32-1	AR Antagonist[7]	Personal Care Products	UK3
4-Chloro-3,5-dimethyl-phenol	Chloroxylenol	88-04-0	AR Antagonist[7]	Personal Care Products	UK1, UK3
Galaxolide 1 and 2 isomer	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-[g]-2- benzopyrane (HHCB)	1222-05-5	AR Antagonist[8]	Personal Care Products	UK1, UK3
Triclosan		3380-34-5	AR Antagonist[7]	Personal Care Products	UK1, UK3
Methyl triclosan		4640-01-1		Personal Care Products /Triclosan Metabolite	UK1, UK3
Isoproturon		34123-59-6	AR Antagonist[9]	Pesticide	UK3
Terbutryn		886-50-0		Pesticide	UK3
Ibuprofen		15307-79-6	Associated with cryptorchidism[10]	Pharmaceutical	UK3
Ibuprofen methyl ester	Methyl 2-(4-isobutyl phenyl) propanoate	61566-34-5		Pharmaceutical Metabolite	UK3
Indomethacin methyl ester		1601-18-19		Pharmaceutical Metabolite	UK3

[1](Selvin *et al.*, 2009), [2](Liu *et al.*, 2009), [3](Ohyama *et al.*, 2005), [4](Honkakoski *et al.*, 2004), [5](Vinggaard *et al.*, 2000), [6](Hawliczek *et al.*, 2012), [7](Rostkowski *et al.*, 2011), [8](Schreurs *et al.*, 2005), [9](Orton *et al.*, 2009), [10](Kristensen *et al.*, 2010).

Table 4.3 also includes a few additional chemicals of interest which were taken forward to *in vitro* screening for confirmation of activity to identify other possible anti-androgenic chemicals. These included methyl triclosan, terbutryn, ibuprofen methyl ester and indomethacin methyl ester. Their screening was justified for a number of different reasons. Whilst the anti-microbial agent triclosan is known to be anti-androgenic, there was no data available for the activity of its metabolite methyl triclosan, which was detected in both effluents. Terbutryn is a pesticide, which has recently been added to the EU's Water Framework Directive as a priority pollutant and therefore warrants assessment since many other pesticides are anti-androgenic (Orton *et al.*, 2011). Finally, maternal use of the pharmaceutical ibuprofen has been linked to cryptorchidism in newborns in an epidemiology study, indicating an anti-androgenic effect (Kristensen *et al.*, 2010). Indomethacin has also been demonstrated to be a steroidogenesis inhibitor and the metabolites of both chemicals therefore warrant assessment (Kristensen *et al.*, 2012). This follows on from the assessments completed in Chapters 2 and 3 to delve further into whether pharmaceuticals could be part of the anti-androgen issue. However, indomethacin methyl ester and 11H-benzo[b]fluorine, which was anti-androgenic in the AR CALUX assay (Hawliczek *et al.*, 2012), were not available for purchase from Sigma-Aldrich and therefore could not be tested in this study. This left a total of 24 standards for purchase, *in vitro* screening and confirmation of activity. Out of these tested chemicals, only five were confirmed as anti-androgenic in the YAS. These are shown in Table 4.4, whilst the yeast screen results are shown in Figure 4.6 and Figure 4.7.

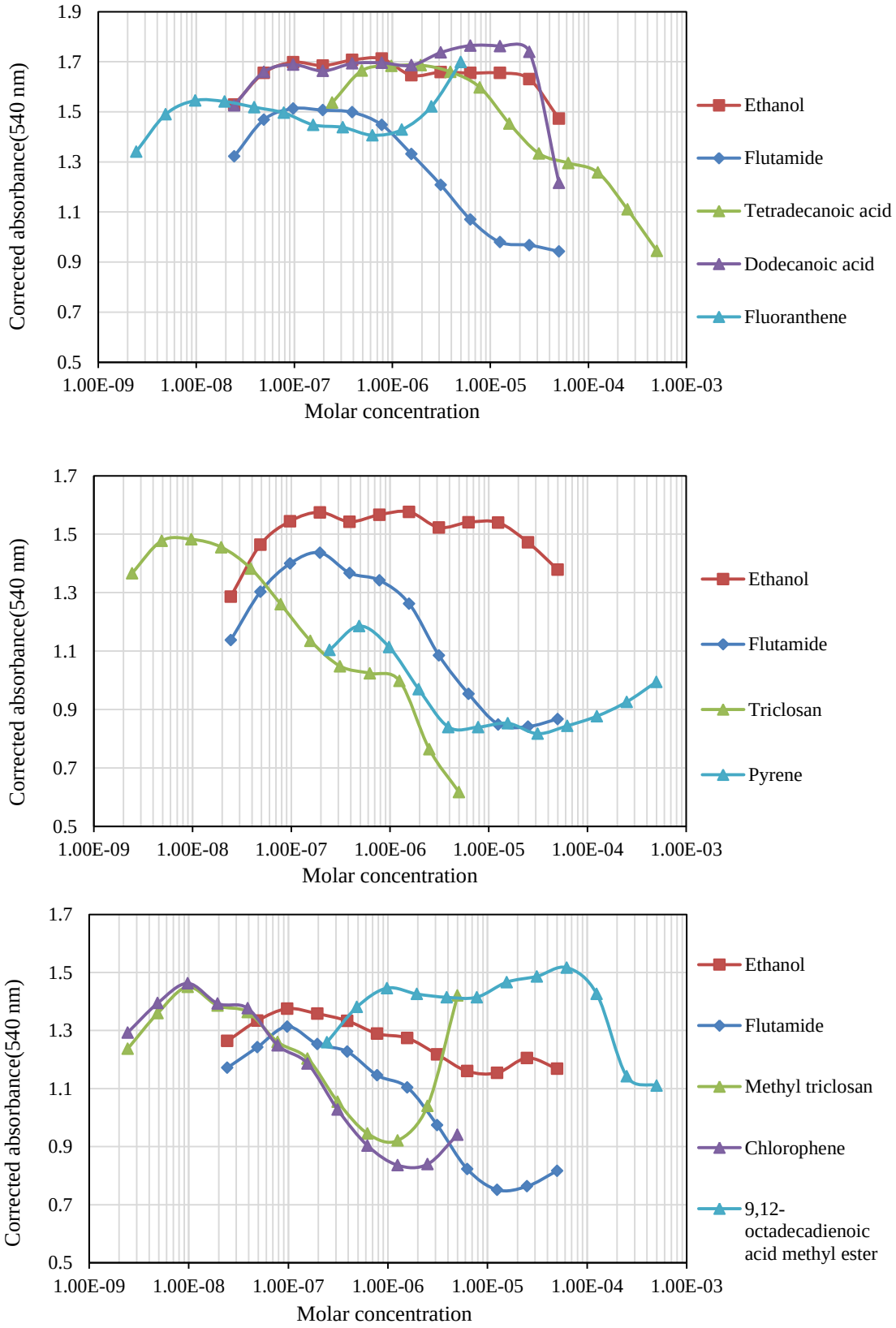


Figure 4.6 *Responses of active chemicals in the yeast screen. Corrected absorbance at 540 nm quantifying colour change.*

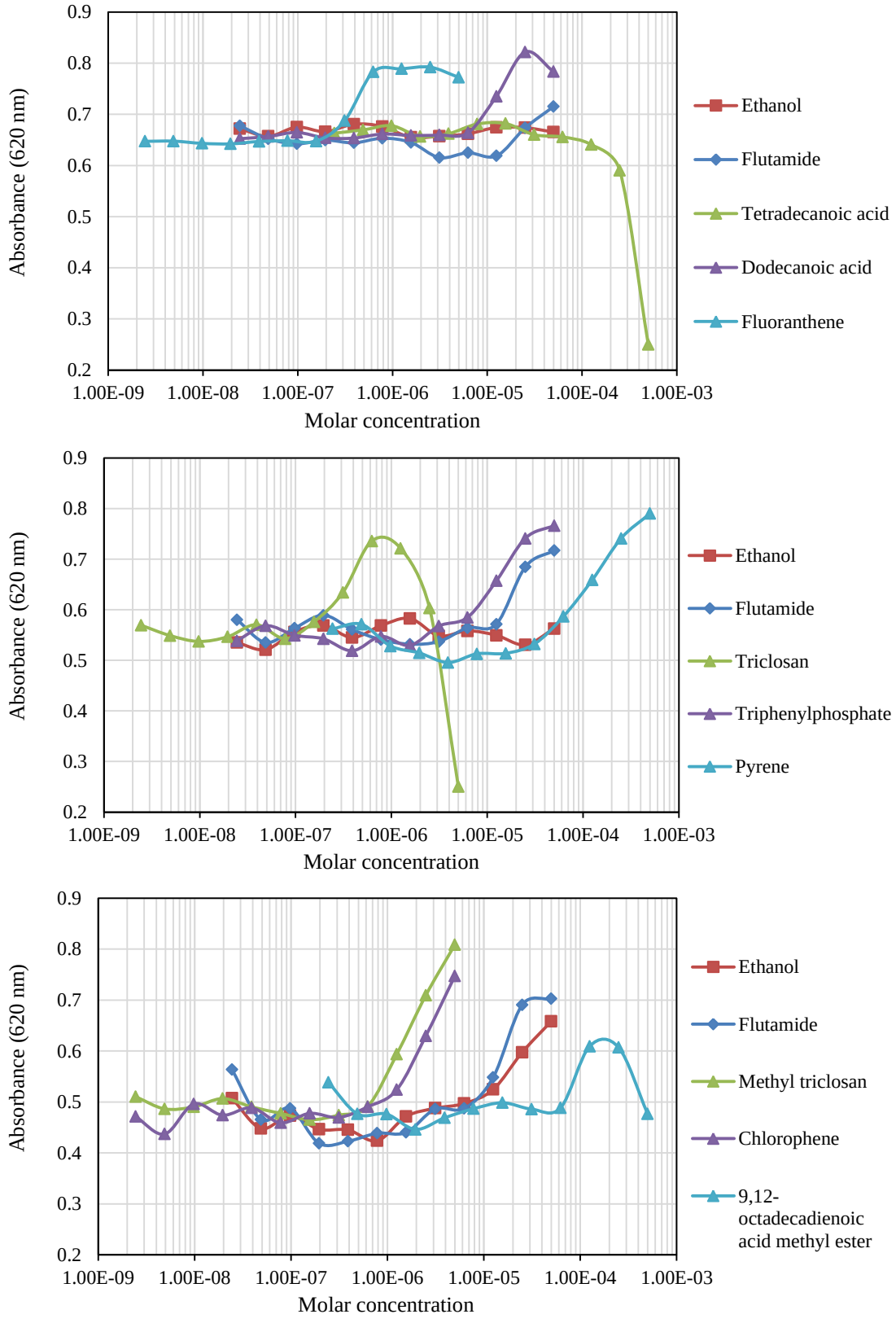


Figure 4.7 *Turbidity readings (620 nm) responses from active chemicals in the yeast screen showing proliferative and toxic effects.*

The confirmed anti-androgens included triclosan, chlorophene, methyl triclosan, pyrene and tetradecanoic acid, in their order of potency. With the exception of tetradecanoic acid, the confirmed anti-androgens were highly potent chemicals, exceeding the potency of flutamide. In comparison, 9,12-octadecadienoic acid methyl ester and dodecanoic acid were only tentatively quantified since full response curves were not achieved. The reduction in absorbance at the highest tested concentrations suggested that they were weakly anti-androgenic, although this should be confirmed in further testing. When the FluEqs and concentrations of confirmed anti-androgens from the samples were assessed, it was found that the total anti-androgenic activity that they could explain was incredibly low. Indeed, the confirmed anti-androgens were only expected to contribute 778 ng/L and 200 ng/L FluEQ in samples from UK1 and UK3 respectively. In comparison, anti-androgenic activity equating to 242 and 325 µg/L was detected in extracted whole effluent samples from UK1 and UK3. As a result, the contribution of the confirmed anti-androgens only equated to 0.08% and 0.24% of the total activity identified at UK1 and UK3. Interestingly, only 9,12-octadecadienoic acid methyl ester, methyl triclosan and pyrene were detected in the active fractions identified during preliminary screening. However, methyl triclosan was identified only in an active fraction in UK3 and not UK1, where its concentration was lower. The contribution of confirmed anti-androgens in both river samples was also very low, since fewer chemicals were detected in these samples. On the Avon, only 18 ng/L FluEQ could be expected from the contribution of decanoic acid and tetradecanoic acid, in comparison to 74 ng/L on the Erewash, due to the presence of pyrene. The turbidity readings were also highly variable between chemicals. Toxicity was observed for tetradecanoic acid and triclosan, which also caused proliferation prior to toxicity. When anti-androgenic activity was quantified for these chemicals, the wells exhibiting toxicity were not used in comparisons to the flutamide curve. For example, tetradecanoic acid was toxic from 0.25 mM ($2.5x10^{-4}$ M) but anti-androgenic from 7.8 µM ($7.8x10^{-6}$ M). In addition, the corrected 540 nm absorbance of methyl triclosan suggested that it was androgenic at high concentrations. However, this could have been a result of its proliferative effect at these concentrations, increasing the cell numbers to increase the 540 nm absorbance. Once again the edge effect was also clearly observed in the solvent controls from the first two screens, with a reduction in the corrected 540 nm absorbance in peripheral wells. As a result, with the exception of 9,12-Octadecadienoic methyl ester and dodecanoic acid, the highest concentration wells were not used to determine the FluEQ. Consequently, the potency of 9,12-Octadecadienoic methyl ester and dodecanoic acid could be slightly overestimated and warrants further assessment.

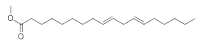
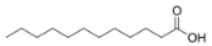
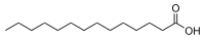
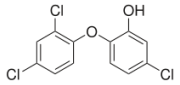
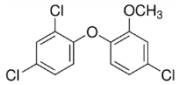
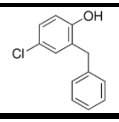
Compound	Structure	Origin	RT	Ions (mz)	Sample	Anti-androgenic fraction?	ng/L	FluEQ	Contribution to total activity
9,12-Octadecadienoic acid methyl ester		Fatty Acid	30.25	67,81,95,109, 263,294	UK1	Y	26	0.003	0.08 ng/L (0.0003%)
					UK3	Y	23		0.07 ng/L (0.00002%)
Dodecanoic acid		Fatty Acid	22.75	74,87,43,55, 214	UK3	N	126	0.002	0.25 ng/L (0.00008%)
					Avon	n/a	2		0.004 ng/L
Tetradecanoic acid		Fatty Acid	25.65	74,87,143,199, 242	Avon	n/a	360	0.05	18 ng/L
Pyrene		PAH	29.88	202,203,200,201, 101	UK1	Y	4	3.5	14 ng/L (0.006%)
					Erewash	n/a	21		74 ng/L
Triclosan		Personal Care Products	30.26	288,290,218,146, 114	UK1	N	13	12.4	161 ng/L (0.07%)
					UK3	N	37		459 ng/L (0.14%)
Methyl triclosan		Personal Care Products	30.44	302,304,252,255	UK1	N	4	6.1	24 ng/L (0.01%)
					UK3	Y	11		67ng/L (0.02%)
Chlorophene		Personal Care Products	27.66	218,183,140,220, 165,152,112	UK3	N	35	7.2	252 ng/L (0.08%)
Contribution of known anti-androgens to the total activity of STW effluent samples UK1: 0.200 µg/L out of 242 µg/L (0.08%) UK3: 0.778 µg/L out of 325 µg/L (0.24%)									

Table 4.4 *Chemicals with confirmed anti-androgenic activity in the YAS. This shows their retention times (RT) and fragment ions from GC-MS analysis as well as their concentrations in the samples. The contribution of each chemical to anti-androgenic activity in a sample is indicated based on its potency relative to flutamide and its concentration. This is displayed as both ng/L FluEQ and the % of the total activity of the whole effluent extracts. Dodecanoic acid and 9,12-Octadecadienoic methyl ester are tentatively quantified and confirmed as anti-androgenic since full response curves were not achieved.*

4.4 Discussion

4.4.1 Identified anti-androgenic chemicals

4.4.1.1 Fatty acids

Lipids and their breakdown products, fatty acids and their methyl and ethyl ester derivatives, are major constituents of domestic WWTW effluents, originating from soaps, food oils and faecal matter following human excretion (Alves *et al.*, 2001; Prats *et al.*, 1999). Since they commonly occur in effluent, fatty acids have been used as markers of WWTW effluent contamination in the aquatic and estuarine environment (Quemeneur and Marty, 1992). Indeed, a suite of fatty acids were identified in this study in effluent and river samples, including pentadecanoic acid, tetradecanoic acid methyl ester and dodecanoic acid. These are of particular interest since they were recently extracted from a fungus, *Ganoderma lucidum* used in traditional medicine and were found to significantly inhibit 5α-reductase derived from fresh rat liver explants (Liu *et al.*, 2009). This tested a suite of fatty acids and found only these saturated fatty acids with a carbon chain length of 12-16 and unsaturated fatty acids with a carbon chain of 18 to be active. Indeed, these results were supported by an independent study which used the same method to test these fatty acids after they were extracted from the plant saw palmetto (*Serenoa repens*) (Abe *et al.*, 2009). Saw palmetto has become popular as an alternative therapy for benign prostate hyperplasia, which can normally be treated with pharmaceutical 5α-reductase inhibitors dutasteride and finasteride. However, a recent meta-analysis of long term clinical trials failed to detect any benefit of using saw palmetto over the placebo (Tacklind *et al.*, 2012). Whilst their 5α-reductase activity could not be confirmed in our laboratory, these chemicals were tested for AR antagonism in the YAS. This only confirmed weak anti-androgenic activity in 9,12-octadecadienoic acid methyl ester, which was considerably less potent than

flutamide. Further analysis of dodecanoic acid is also warranted to confirm its anti-androgenic activity, since a reduction in absorbance was observed at its highest tested concentrations but a full response curve was not achieved. In comparison, hexadecanoic acid methyl ester and 9-Octadecenoic acid methyl ester were reported as anti-androgenic based on Dr Dukes Phytochemical and Ethnobotanical Database (Selvin *et al.*, 2009). However, examination of this database yielded no evidence to support its assertion that these chemicals were anti-androgenic and failed to cite or demonstrate any work that tested this statement. No additional data was available in the primary literature to support this assertion. As a result, with the exception of 9,12-octadecadienoic acid methyl ester and possibly dodecanoic acid, there is little evidence to suggest that these chemicals are AR antagonists. Nonetheless, the identification of some of these natural chemicals as 5α-reductase inhibitors (Liu *et al.*, 2009) contradicts the assumption that anti-androgenic chemicals in the environment are limited only to synthetic chemicals.

4.4.1.2 Phosphorous flame retardants

Phosphorous flame retardants are chemicals which are added to polymers to prevent combustion or to delay the spread of fire after ignition (Kemmlein *et al.*, 2003). They have become more popular in recent years as alternatives to brominated flame retardants, which have seen greater restrictions in the EU and under the Stockholm Convention. Since they are not chemically bonded to the final product, they can leach out and enter the environment through a number of routes, including WWTW effluent (Rodriguez-Mozaz *et al.*, 2007). Concentrations in surface water of up to 379 ng/L have been detected, although concentrations in µg/L range have been observed in WWTW effluents (van der Veen and de Boer, 2012). As a result, these chemicals are widespread contaminants of the aquatic environment, as evidenced by a survey of US rivers, which found trischloroethyl phosphate to be one of the most frequently detected chemicals (Kolpin *et al.*, 2013). This study identified tri (butoxyethyl) phosphate, Tri-n-butyl phosphate, triphenyl phosphate and trischloroethyl phosphate as known anti-androgens, based on the MDA-kb2 breast cancer cell line (Ohyama *et al.*, 2005). However, these chemicals were found to be inactive in the YAS. There are no further reports of anti-androgenic activity from these chemicals. However, trischloroethyl phosphate, also known as tri (2-chloroethyl) phosphate, has gained regulatory interest in the European Union (EU) under REACH and has recently been classified as a substance of high concern due to its reproductive toxicity (ECHA, 2009). Indeed, decreased sperm motility, sperm density and testes weight, as well as increases in

sperm abnormalities were observed in mice exposed to tri (2-chloroethyl) phosphate (Beth-Hübner, 1999). However, it is not known whether this is through an anti-androgenic mode of action.

4.4.1.3 Personal care products

Like pharmaceuticals, a wide range of chemicals originating from personal care products have been detected in effluents and surface water, following human use, disposal and manufacturing (Boxall *et al.*, 2012). They include UV screens, insect repellents, surfactants and other constituents of cleaning products, as well as fragrance chemicals and cosmetic products (Caliman and Gavrilescu, 2009). Many are in widespread use and as a result, some are high production chemicals, such as linalool, which is used as a fragrance agent in cleaning products. Others, such as caffeine, are constantly released by the human population and can therefore be seen as pseudo persistent chemicals in the receiving environment (Weigel *et al.*, 2002). Of particular note in this study was the detection of the synthetic musk galaxolide (HHCB) and the biocides chlorophene, chloroxylenol, triclosan and its metabolite methyl triclosan. With the exception of methyl triclosan, these chemicals were detected in anti-androgenic fractions from fish bile and confirmed as anti-androgenic in the YAS and AR CALUX assays by Rostkowski *et al*. (2011) This study found triclosan and chlorophene to be significant contributors, accounting for 51% of the total anti-androgenic activity in fish bile (Rostkowski *et al.*, 2011). Our study also found triclosan and chlorophene to be the most highly active anti-androgens detected by GC-MS in the fractions, exceeding the potency of flutamide. However, they were unlikely to be significant contributors to total anti-androgenic activity due to their low concentrations. In further contrast to Rostkowski *et al.,* (2011) this study found that triclosan was more potent than chlorophene. In addition, methyl triclosan was found for the first time to be anti-androgenic with a FluEQ of 6.1. However, chloroxylenol was highly toxic in our assay, to the extent that anti-androgenic activity could not be confirmed. In addition, no activity was detected for HHCB, which was identified during analysis of anti-androgenic fractions from river sediment in a previous study and found to be active in the AR CALUX assay (Weiss *et al.*, 2011; Schreurs *et al.*, 2005). This could have been a result of incompatibility with the yeast screen, possibly due to a lack of permeability of the cell wall for this chemical, which would not have occurred in the mammalian CALUX line. Further assessment of HHCB, using the solvent dimethyl sulfoxide instead of ethanol, could be completed since this can increase cell wall permeability (Beresford *et al.*, 2000).

There is very little literature on the presence of chlorophene in the aquatic environment or its effects in fish. However, triclosan has been subject to considerably more research. It has been detected in effluents and surface water globally, as well as fish bile, human urine and breast milk (von der Ohe *et al.*, 2012; Allmyr *et al.*, 2006; Houtman *et al.*, 2004; Kolpin *et al.*, 2002a). It is in high volume use and originates from soaps, toothpastes and other personal care products, where it is used as an anti-septic. There have been few studies of its effects in fish, although so far there is no evidence to indicate that exposures to environmentally relevant concentrations can cause adverse effects. Indeed, paired medaka showed no differences in egg production or fertility in exposures up to 200 µg/L, although hepatic vitellogenin was induced at 20 µg/L, where embryo hatching was also adversely affected (Ishibashi *et al.*, 2004). Similarly, impairment to swimming behaviour was observed in fathead minnows larvae exposed to 75 µg/L (Fritsch *et al.*, 2013). In comparison, effluent concentrations are reported from 35 ng to 2.7 µg/L (reviewed in Ying and Kookana, 2007) and so testing of environmentally relevant concentrations is warranted. So far the anti-androgenic activity for triclosan has not yet been confirmed *in vivo*. However, much more concerning is the impact on microalgae, which have been identified as the most sensitive species to triclosan, with a predicted no effect concentration for acute toxicity of 4.7 ng/L, well within the environmental range (von der Ohe *et al.*, 2012). As a result of its toxicity and its widespread contamination to the aquatic environment, wildlife and humans, there have been recent calls for regulatory action against triclosan (Halden, 2014).

4.4.1.4 Pesticides

Pesticides, such as DDT and vinclozolin, were amongst the first environmental chemicals to be identified as anti-androgens, causing reproductive tract malformations in male rodent models (Kelce *et al.*, 1998). Similar effects have also been observed in fish models, where reductions in sperm count, testes size, secondary sexual characteristics were observed alongside disruption to courtship behaviour (Baatrup and Junge, 2001). These chemicals have both agricultural and non-agricultural uses, entering the environment through runoff. However, WWTW effluent can also be a source, as evidenced by the presence of terbutryn and isoproturon in the UK3 effluent. Both of these chemicals are now part of the EU's Water Framework Directive as priority pollutants and isoproturon was withdrawn from the market in 2009. Although many of these chemicals have been banned or restricted in some countries, they are still detected in the aquatic environment (Baugros *et al.*, 2008). It has been well known for

a number of years that many pesticides in current use have endocrine disrupting properties and recent studies have found that many pesticides are anti-androgenic. Indeed, a study of endocrine activity in *in vitro* reporter gene assays using Chinese hamster ovary cell lines transfected with the human AR found that 66 out of the 200 tested pesticides were anti-androgenic (Kojima *et al.*, 2004). Of particular concern is the presence of this activity in a variety of current use pesticides, many of which are detected in the environment (Orton *et al.*, 2011; Aït-Aïssa *et al.*, 2010; Orton *et al.*, 2009). Out of these chemicals, isoproturon was the only known anti-androgenic pesticide detected in this study, in the UK3 fractions. However, its anti-androgenic activity was not confirmed at the concentrations employed in this study and it might be that greater concentrations are required to illicit a response in the yeast screen. Indeed, Orton *et al.* (2009) detected anti-androgenic activity for isoproturon in the YAS at 250 µM in comparison to the 0.5 µM tested in this study to avoid toxicity (Orton *et al.*, 2009). Similarly, no activity was observed in a separate study employing the MDA-kb2 breast cancer cell line at 1 and 10 µM (Aït-Aïssa *et al.*, 2010). Subsequent analysis of the chemicals detected by GC-MS identified additional pesticides, including 1,3,5,Triazine-2,4,6(1H,3H,5H)-trione, 2,3,4 Trichlorophenol and 2-Ethylhexyl diphenyl phosphate, as well as the herbicide 4-Chloro-2-methylphenoxy acetic acid. As a result, further assessment is warranted to analyse these chemicals, as is further testing of the activity of isoproturon at higher concentrations.

4.4.1.5 Pharmaceuticals

GC-MS analysis tentatively identified a set of pharmaceuticals in the WWTW effluent samples but not at the upstream river sites. They included the non-steroidal anti-inflammatory inhibitors (NSAIDs) ibuprofen, naproxen and the diclofenac metabolite, diclofenac methyl ester. Based on the NHS prescriptions cost analysis for 2009, these were amongst the top 100 prescribed pharmaceuticals in the UK (The NHS Information Centre, Prescribing Support Unit, 2010). In addition, indomethacin methyl ester, the metabolite of another NSAID with a lower prescription rate was also detected. Out of this set of pharmaceuticals, indomethacin methyl ester and ibuprofen were of particular interest as potential anti-androgens. NSAIDs have recently gained significant interest from the human health perspective since the use of ibuprofen, asprin and another analgesic, paracetamol, by pregnant women was associated with congenital cryptorchidism in male offspring in a dose dependant manner (Kristensen *et al.*, 2010). Cryptorchidism is indicative of anti-androgenic demasculinisation and has been associated with a number of anti-androgenic chemicals in mammalian studies (Gray *et*

al., 2001). However, it was also been suggested that this could be a result of prostaglandin synthesis inhibition by these chemicals. Prostaglandins have been shown to play a role in male development, inducing sertoli cell differentiation from somatic cells under the regulation of testosterone, demonstrating a role in the action of androgens (Adams and McLaren, 2002; Gupta, 1989). Indeed, ibuprofen is the most potent prostaglandin synthesis inhibitor of these chemicals and was associated with the highest risk of congenital cryptorchidism (Kristensen *et al.*, 2010). Indomethacin was also found to have anti-androgenic effects in both foetal rat and adult human testes explants, where testosterone production was reduced (Albert *et al.*, 2013; Kristensen *et al.*, 2012). This suggests that it could be acting as a steroidogenesis inhibitor to exert these effects, but the exact mechanism is still subject to research and indomethacin methyl ester was not available for testing in this study. In addition, YAS screening of ibuprofen and its metabolite found no evidence of anti-androgenic activity, supporting the possibility of its anti-androgenic action through steroidogenesis inhibition. The other pharmaceuticals detected in this study, carbamazepine, phenobarbital, primidone and ethosuximide, are all used as anti-convulsants and range in their prescriptions. However, there was no previously reported anti-androgenic activity for these chemicals.

The anti-androgenic pharmaceuticals targeted by predictive modelling in Chapter 2 were not detected by this analytical technique, although they were predicted to be present in the effluents of UK1 and UK3. GC-MS depends on the volatisation of chemicals to a gaseous form for separation prior to analysis by mass spectrometry. It is likely that bicalutamide and cyproterone acetate were not possible to volatise during this process since they have higher molecular weights and boiling points than the pharmaceuticals that were successfully detected. However, the presence of pharmaceutical chemicals which can act as steroidogenesis inhibitors demonstrates that bicalutamide and cyproterone acetate are not the only pharmaceuticals in the environment which can potentially disrupt the androgen signalling pathway.

4.4.1.6 Polyaromatic hydrocarbons

PAHs are ubiquitous, persistent pollutants originating from incomplete combustion, although some are also high production chemicals, such as pyrene (WHO/UNEP, 2013). Some, including benzo[a]pyrene, are listed under the Water Framework Directive as high priority substances due to their toxicity and mutagenicity (Brack *et al.*, 2007). They are widespread in the environment and are also commonly detected in the

human body (Woodruff *et al.*, 2011). Benzo[a]pyrene, chrysene, pyrene and fluoranthene were found to be anti-androgenic in Chinese hamster ovarian cell lines, transfected with a transient AR (Vinggaard *et al.*, 2000). Here, benzo[a]pyrene was the most potent chemical, whilst pyrene was only shown to be moderately active. This contrasts with findings from this study, which only confirmed activity in pyrene, which was found to be more potent than flutamide. However, another *in vitro* assessment of PAHs, has suggested alternative mechanisms for their anti-androgenic responses. Here, PAHs which were anti-androgenic in the LNCaP human prostate cancer cell line did not to bind to the AR itself. However, they were aryl hydrocarbon receptor (AhR) agonists and it was suggested that this caused activation of the transcription factor activator protein-1 (AP-1), which interacted with the AR to inhibit its binding to the androgen response element (Kizu *et al.*, 2003a). Indeed, by adding an AhR antagonist the anti-androgenic response from the cell line was reversed. In agreement, the study by Vinggaard *et al.,* found that some of the most potent AR antagonists were also the most potent AhR agonists (Vinggaard *et al.*, 2000). These chemicals included Benzo[a]pyrene and chrysene, but not pyrene, which was inactive in the LNCaP cell line, suggesting that there may be differences in assay sensitivity between mammalian LNCaP cells and the YAS for this chemical (Kizu *et al.*, 2003a). Indeed, pyrene was also found to be inactive in the mammalian AR CALUX assay after it was identified in anti-androgenic fractions of extracted sediment from Belgium (Weiss *et al.*, 2009).

4.4.2 Contributions to environmental anti-androgenic activity

Whole effluent extracts from UK1 and UK3 were found to contain 242 µg/L and 325 µg/L FluEQ respectively, which compare well with anti-androgenic activity detected in a UK survey of 43 WWTWs that ranged from 21.3 to 1,231 µg/L FluEQ (Johnson *et al.*, 2007b). However, the confirmed anti-androgens detected in the fractionated samples made very minor contributions to the total activity of extracted whole effluent samples. Although some were very potent anti-androgens, the low concentrations at which they were present meant that they could only explain 0.08% and 0.24% of the total activity in UK1 and UK3. This concurs with previous studies of anti-androgenic activity in complex environmental samples where targeted analysis has only been able to explain a small proportion of the measured activity (Kinani *et al.*, 2010; Urbatzka *et al.*, 2007). A majority of the confirmed anti-androgens were not found in active fractions; further indicating the presence of other chemicals which this EDA was unable to detect. Since activity was diluted throughout a number of fractions, it is likely that the anti-androgenic activity detected originated from a range of chemicals with a range of chemical

properties. However, further analysis is required to properly identify these chemicals, which will be further discussed.

It should be considered that assessing chemicals for AR antagonism through the use of bioassays will not capture 5α-reductase inhibitors, which were identified in this study by literature review. These chemicals can reduce the conversion of testosterone to DHT through enzyme inhibition of the two 5α-reductase isoforms and can cause anti-androgenic effects through this mechanism. DHT is a more potent androgen than testosterone and plays an important role in sexual development and the regulation of secondary sexual characteristics in vertebrates (Langlois *et al.*, 2010). However, it is only recently that DHT was found to be a physiologically important androgen in fish, where previously it was assumed that testosterone and 11-ketotestosterone were the only functional endogenous androgens (Margiotta-Casaluci and Sumpter, 2011). As a result, the presence of 5α-reductase inhibitors, or indeed this activity in effluent, has received little attention from the perspective of endocrine disruption in fish. Nonetheless, DHT has now been detected in fish, where it is produced mainly in the testes in fathead minnows, and 5α-reductase enzymes have been sequenced in zebrafish (Margiotta-Casaluci *et al.*, 2013a; Martyniuk *et al.*, 2013). *In vivo*, DHT was found to have a similar potency to 11-ketotestosterone when increasing somatic growth, inducing secondary sexual characteristics and disrupting ovarian morphology, to the extent that testicular tissue was observed in females (Margiotta-Casaluci and Sumpter, 2011). Consequently, physiology and reproductive capacity can be affected in fish exposed to 5α-reductase inhibitors, with the pharmaceutical dutasteride causing a reduction in fecundity and secondary sexual characteristics in fathead minnows (Margiotta-Casaluci *et al.*, 2013b). The effects of these chemicals are better characterised in mammalian models, where they have been shown to disrupt reproductive development in males, inducing hypospadias, nipple retention and reducing ano-genital distance (Bowman *et al.*, 2003). In addition, when incorporated in a mixture with AR antagonists and testosterone synthesis inhibitors, additive effects and even synergistic effects were observed in endpoints relating to sexual development in rats (Christiansen *et al.*, 2009). As a result, 5α-reductase inhibitors warrant further research due to their potential impacts in fish and their environmental presence.

4.4.3 Future considerations for effect directed analysis of environmental anti-androgens

It is clear that further assessment is required to better characterise anti-androgenic chemicals in environmental samples. However, as this study has found, the task presents a significant challenge. WWTW effluents are complex samples due to the large numbers of chemicals present, including metabolites and breakdown products, of which many remain uncharacterised. Consequently, EDA still remains a sensible tool for use in identifying and targeting chemicals of interest from the plethora available in the environment. However, the method needs to be adapted to improve the efficacy of this type of assessment for identifying anti-androgens. The results of this study have provided a number of insights, which can be used to inform and drive modifications in the EDA method. These are discussed below and include changes to sample collection and extraction, fractionation, direction of analysis with *in vitro* bioassays and the final chemical identification stage.

It was clear from the fractionation procedure that the anti-androgenic activity detected in the whole effluent sample was diluted out between a number of fractions, indicating that chemicals with a range of chemical properties were contributing. As a result, only partial dose response curves were achieved when the fractions were tested in the YAS. This could also be a result of inevitable chemical loss or degradation during the EDA process, resulting in a low recovery of the total activity of the effluent samples. Indeed, failure of SPE to capture or elute chemicals and adherence to glassware during sample concentration would result in chemical loss. To mitigate against low recovery and spread of chemicals throughout the fractions, extraction of greater volumes than the 2.5 L samples collected in this study could be employed. Indeed, previous studies employing EDA have tended to use large volume extractions. When the steroid oestrogens were first detected in WWTW effluent fractions a 20 L volume had been extracted (Desbrow *et al.*, 1998). In comparison, other studies of anti-androgenic activity using EDA began by extracting 10 L of river water samples from Italy, whilst Thomas *et al.* (2009) extracted 50 L of offshore produced water (Thomas *et al.*, 2009; Urbatzka *et al.*, 2007). This would also increase concentrations of chemicals in fractions for yeast screening, making them more likely to produce full response curves to improve the direction of analysis. However, based on the spread of activity in the fractions from this study, there is the potential that a large number of chemicals at low concentrations could be contributing to the total activity and producing a “something from nothing” effect. As a result, some chemicals may not all be present in active fractions, as was observed with the potent anti-androgens, triclosan and methyl

triclosan, during this study. Future analysis should therefore assess the percentage of the total activity recovered in active fractions, by combining active fractions and retesting the composite sample in the YAS. Unfortunately, due to limited sample volume this could not be completed as part of this study.

Producing fractions from higher sample volumes may induce toxicity during yeast screening, which could mask anti-androgenic activity, as a result of the increased chemical concentrations. Arguably, the androgenic activity identified in the fractions in this study could also have masked the presence of some chemicals. Indeed, one of the more potent anti-androgens, chlorophene, was detected in an androgenic fraction in UK1. These masking effects could be avoided through a greater degree of separation, which could be achieved by two-dimensional liquid chromatography. This involves the application of two independent separation systems to a sample, which significantly increases peak capacity (the resolving power) over the one dimensional chromatography used in this study (Stoll *et al.*, 2007). Nonetheless, this would produce a greater number of fractions for *in vitro* analysis.

In vitro bioassays still have the potential to play a key role in directing an EDA for AR antagonists, to reduce the number of chemicals for screening and to better identify causal chemicals. Indeed, they have been used successfully in a range of similar studies identifying endocrine disruptors following effluent fractionation (Rostkowski *et al.*, 2011; Weiss *et al.*, 2009; Urbatzka *et al.*, 2007; Desbrow *et al.*, 1998). However, a number of confounding factors were observed during this study, which should be considered when developing and interpreting EDA approaches to ensure adequate direction and confirmation is achieved. Firstly, the edge effect was observed in some plates when samples were tested. Here, a decrease in 540 nm absorbance occurred in the peripheral wells of the plate to produce a shallow U shaped curve. This is a known factor responsible for reducing assay performance in *in vitro* screens, which has led some researchers to avoid using the peripheral wells at the cost of throughput. It can be caused by thermal gradients across plates during incubation and one study showed that this could be reduced by leaving plates at room temperature prior to incubation (Lundholt *et al.*, 2003). The second factor affecting the absorbance was the alterations in turbidity caused by proliferative and toxic effects of the chemical standards on the yeast cells during confirmation of activity. It was clear that 540 nm values could not be sufficiently corrected and highlighted the importance of assessing turbidity alongside the AR mediated response to explain the observed effects. This was particularly evident for methyl triclosan, where an increase in turbidity was associated with an increase in corrected 540 nm absorbance, which alone would otherwise suggest

androgenic activity. Toxicity was also evident in the chemical standards, which exacerbated the 540 nm absorbance readings. Interestingly, flutamide was also shown to be toxic, although this varied between assays even though it was tested at the same concentration throughout the study. This has been observed previously by Orton *et al.,* who switched to using the anti-androgenic pesticide, procymidone, as a positive control, since it was nontoxic to the MDA-kb2 cell line (Orton *et al.*, 2011). This is presently being tested in the YAS at Brunel to assess toxicity and anti-androgenic activity for its potential use as a positive control. In the case of the chemical standards, wells where toxicity was observed were not used to determine the potency of the chemical. However, it is plausible that chronic effects could be occurring in the cells below the concentrations at which acute toxicity was observed. This could have affected the production of enzyme β-galactosidase and could have in turn impacted the 540 nm absorbance. As a result, the anti-androgenic activity of some chemicals could have been overestimated, which could explain why the toxic triclosan was more potent than nontoxic chlorophene, in contrast to *in vitro* screening of anti-androgens by Rostkowski *et al.* (2011). The possibility of chronic effects on cellular machinery is presently being tested at Brunel with the use of mitochondrial assays, such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Van Meerloo *et al.*, 2011).

Following fractionation and *in vitro* analysis, this study employed GC-MS and a mass spectral library to tentatively identify chemicals within the samples. However, this only identified 15-30% of the chemicals detected by GC-MS, the rest were unknown. It should be considered that the use of GC-MS in identifying chemicals is limited to nonpolar and medium polarity chemicals, which can evaporate without degradation. As a result, polar compounds, ionic compounds and those with higher molecular weights, such as some pharmaceuticals and personal care products, are less effectively analysed (Burgess *et al.*, 2013). This was clearly an issue during this study, where few confirmed anti-androgens were found in the active fractions, showing that chemicals were being overlooked by this analytical technique. This suggests that further fractionation of active fractions to target anti-androgens during this study would not have been an effective means of further analysis, if GC-MS was used to detect and identify chemicals.

To improve the detection and identification of chemicals, alternative instrumentation could be employed. Some recent research has employed LC-MS to identify individual chemicals in complex mixtures. However, at present the spectral libraries associated with these techniques are not as extensive as those used for GC-MS, although they

are being developed. Alternatively, methods of identification using more sensitive analytical procedures, such as time of flight mass spectrometers and the Orbitrap have been proposed for use. Indeed, a high-resolution LTQ-Orbitrap has been used in an EDA for anti-androgens and identified a set of eight chemicals, including an oxygenated polyaromatic hydrocarbon, organophosphates, musks, and steroids (Weiss *et al.*, 2011). Time of flight mass spectrometers and the Orbitrap both have a higher mass accuracy and resolution than standard mass spectrometers, combined with high full spectrum sensitivity, which increases the identification reliability when proposing structures (Jernberg *et al.*, 2013; Zwiener and Frimmel, 2004). They can also be used alongside accurate mass databases to identify specific chemicals in a similar way to the use of spectral libraries with GC-MS (Becue *et al.*, 2011). However, there are significantly higher costs associated with operating these instruments, which were outside of the budget for this project.

A final consideration is the environmental media analysed by the EDA process. The bile of fish exposed to WWTW effluent can be used to analyse compounds that are bioavailable. Unlike analysis of effluents, this has the advantage of only assessing compounds that are uptaken by fish, which might be more relevant to any biological effects observed. In addition, some compounds bioconcentrate in bile, bringing their concentrations above LODs of analytical instruments which may exceed effluent concentrations. This was the case with the equine oestrogen metabolite, 17β-dihydroequilenin, which was bioconcentrated in bile from a pictogram to low ng/L concentration in effluent (Gibson *et al.*, 2005). Whether this is the case for triclosan, as a significant contributor to activity in bile of effluent exposed fish but not our tested effluents, remains to be determined (Rostkowski *et al.*, 2011). Nonetheless, characterising the contents of WWTWs effluents to determine what compounds are released into the environment remains an important line of research. Particularly given that inter and intra species differences in uptake, bioconcentration, metabolism and elimination of compounds has been documented (Gibson *et al.*, 2005; Tyler *et al.*, 2005).

4.4.4 Conclusions

EDA was used to identify chemicals contributing to ant-androgenic activity in WWTW effluents and river water samples through the use of fractionation and the YAS screen. Literature searches were employed to identify known anti-androgens, of which of 26 were identified for further screening. Of these chemicals, five were confirmed as anti-

androgenic in the YAS and two were tentatively identified. These had a wide range of origins and included fatty acids, personal care products, pesticides and PAHs. Some, such a triclosan, were highly potent chemicals but due to their low concentrations, the confirmed anti-androgens were only considered to make a minor contribution to the total anti-androgenic activity (<1%). In addition, only two were detected in active fractions from the effluent samples. It was clear that the issue of anti-androgenic activity in the environment encompasses chemicals beyond the known anti-androgens detected by broad scan GC-MS. More work is therefore required to characterise additional chemicals in effluent using more advanced instrumentation to improve the detection and identification of the as yet unknown contaminants.

CHAPTER 5: GENERAL DISCUSSION AND FUTURE PERSPECTIVES

5.1 Summary

Significant progress was made in achieving the overall project aim. To summarise, a set of anti-androgenic chemicals with diverse origins were identified in environmental samples through predictive modelling and effect directed analysis (EDA). However, they were expected to only make a minor contribution to total anti-androgenic activity identified in the samples. More work is therefore required to further characterise environmental anti-androgens and to identify significant contributors to this activity. An EDA of South Australian WWTWs effluents is also still currently underway for comparison with the UK scenario. The anti-androgenic pharmaceuticals were assessed *in vivo* and found to have no effect on sexual disruption in fish at environmentally relevant concentrations, alone or in combination with steroid oestrogens. However, this does not mean that the plethora of anti-androgenic chemicals present in rivers that make up the measured activity do not have such effects on wild fish in an environmental scenario. In comparison, mixtures of steroid oestrogens induced an environmentally relevant incidence and severity of intersex at concentrations comparable with those observed in rivers and WWTW effluents. This supports the possibility that steroid oestrogens are major drivers of intersex in wild fish. In addition, the use of hydrological modelling predicted that steroid oestrogen concentrations in the environment are likely to increase in the future, in the first assessment of future trends in concentrations of these chemicals in surface water. The progress in identifying and assessing anti-androgens and possible future directions are further discussed below.

5.2 Identifying environmental anti-androgens

This thesis project employed two methods to identify likely anti-androgenic contaminants. The first method employed was predictive modelling of anti-androgenic pharmaceuticals, on the basis that one of the potent environmental oestrogens, 17α-ethinylestradiol (EE2), is an active pharmaceutical ingredient (Caldwell *et al.*, 2012). In the absence of any previous recording of these chemicals in the UK and Australia, this study predicted that anti-androgenic pharmaceuticals were likely to occur in UK and South Australian effluents and rivers in low ng/L concentrations. Differences between countries were also predicted due to their differential prescriptions. Further hydrological modelling, to put exposure concentrations used in chapter three in context, also found that contamination of river catchments across England and Wales by bicalutamide and cyproterone acetate was likely to be widespread. However, the median concentrations were expected to be below 10 ng/L for both chemicals.

The second, broader approach employed EDA to detect anti-androgenic chemicals in UK effluents and river samples. This part of the study employed broad scan GC-MS alongside chemical identification with mass spectral libraries and literature searching to identify known anti-androgens. These included fatty acids, flame retardants, polyaromatic hydrocarbons, personal care products and pesticides. In total 24/26 compounds underwent *in vitro* screening with the yeast androgen screen, of which triclosan, chlorophene, methyl triclosan, pyrene and tetradecanoic acid, were confirmed as anti-androgenic. With the exception of tetradecanoic acid, these were highly potent chemicals, exceeding the potency of flutamide. In addition, 9,12-octadecadienoic acid methyl ester and dodecanoic acid were tentatively identified as anti-androgens since full dose response curves were not achieved in this study. Nonetheless, when the potencies and concentrations of these chemicals were combined, it became clear that they were still likely to make a very minor contribution to the total anti-androgenic activity observed in the WWTWs effluents sampled. A similar EDA process was also employed using effluents from three of the South Australian WWTW's involved in the modelling analysis in chapter one. However, work is still underway to tentatively identify the chemicals in the active fractions from these samples. As a result, although work will continue to analyse these samples, at present the question of the identity of anti-androgenic chemicals in Australian effluents and their potential contribution to total activity remains unanswered by this thesis.

The question of which contaminants are significant contributors to anti-androgenic activity is a complex one, which needs to take into account both concentration and *in vitro* potency. Indeed, this study has shown that the relative contribution of high potency chemicals, such as triclosan and chlorophene, was limited by their low ng/L concentrations. This is also likely to be the case for the anti-androgenic pharmaceuticals, modelled in chapters two and three, which were predicted to occur in the low ng/L range. However, screening of the pharmaceuticals found bicalutamide to be repeatedly inactive in the YAS when serially diluted from 1 mM, whilst cyproterone acetate had a reproducible androgenic profile (Figure 5.1). This suggests that they are unlikely to contribute to the anti-androgenic activity measured in environmental samples by yeast screen. Since bicalutamide is a pure AR antagonist, as indicated by preclinical trials with mammalian cell lines (Furr and Tucker, 1995), this response may be an issue of cell wall permeability within the yeast screen. Indeed, bicalutamide is anti-androgenic in a number of *in vitro* bioassays and has been used as a positive control in an inter-laboratory comparison of four different AR based mammalian cell lines (Körner *et al.*, 2004). Interestingly, bicalutamide was also shown to poorly

penetrate the blood-brain barrier in tissue distribution studies in rats. This explained the peripheral selectivity observed in rats treated with bicalutamide, which was not observed in studies of flutamide (Furr and Tucker, 1995). In comparison, there is *in vivo* evidence of androgenic effects of cyproterone acetate, which is a partial agonist and was shown to cause a 60% increase in the prostate weight of castrated rats following exposure (Poyet and Labrie, 1985). This would explain the induction of a maximal response on top of the DHT background in the yeast screen at the concentrations tested.

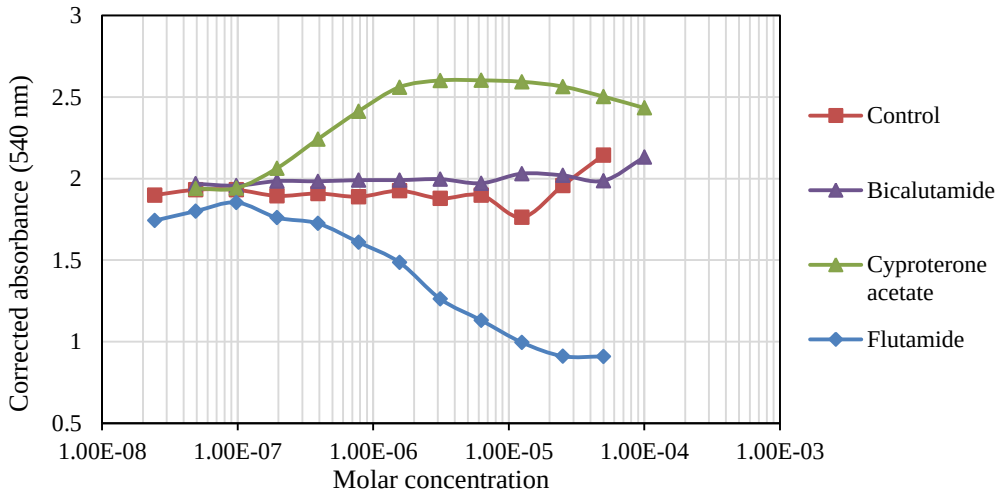


Figure 5.1 *Bicalutamide and cyproterone acetate tested in the YAS, serially diluted from 1 mM with a background of 2 nM DHT.*

If the situation with anti-androgens is similar to that of the steroid oestrogens, where a few chemicals explain the vast majority of oestrogenic activity, concentrations of the as yet unknown, significant contributors would have to be in µg/L range. This could explain the hundreds of µg/L flutamide equivalent activity which has been quantified frequently in environmental samples (Johnson *et al.*, 2007b). These chemicals would also need to be common contaminants of WWTW effluent and are likely to have a human origin in their use due to their frequent association with domestic effluents (Johnson *et al.*, 2007b). Alternatively, a much larger number of chemicals could be acting additively at lower concentrations to cause this response *in vitro* and the circumstances could even be similar to the something from nothing effect documented for oestrogenic EDCs (Brian *et al.*, 2005; Silva *et al.*, 2002). This thesis also suggests that significant contributors to *in vitro* activity in effluents may differ from those associated with effects in fish. Indeed, whilst triclosan and chlorophene are only likely to play a minor role in

the total anti-androgenic activity in effluent (<1%), they were found to explain 51% of the total activity detected in fish bile, where they had accumulated (Rostkowski *et al.*, 2011). As a result, to better understand the risk posed by anti-androgens to fish health, further analysis of the possible *in vitro-in vivo* differences driven by their different pharmacokinetic interactions is required.

In agreement with previous studies, it is clear from these results that currently known anti-androgens identified in environmental samples cannot explain the anti-androgenic activity detected (Kinani *et al.*, 2010; Urbatzka *et al.*, 2007). Further work is required to better characterise the causal chemicals and, although the methodology encountered difficulties, EDA remains suitable for this assessment. Indeed, starting with a greater extraction volume will benefit the *in vitro* screening process in accurately identifying active HPLC fractions, although biological screening should still be scrutinised for confounding factors, particularly toxicity. With better direction of analysis, it will be easier to reduce the number of chemicals required for identification and assessment out of the thousands found in WWTW effluent. In addition, as more chemicals are identified as known anti-androgens, it may become easier to identify contributors to the total activity in environmental samples. Indeed, preliminary results from the Tox21 program found that almost 10% of the 1,462 chemicals tested were anti-androgenic androgen receptor (AR) antagonists *in vitro,* which supports the possibility of a large number of contributors to environmental activity (Tice *et al.*, 2013). These are only the results from initial phase I testing in a US collaboration aiming to screen thousands of chemicals, associated with human exposure, for toxicity. The next stage of screening incorporates a library of over 10,000 chemicals including industrial chemicals, sunscreen additives, flame retardants, pesticides, plasticizers, solvents, food additives, natural product components, drinking water disinfection by-products, preservatives, therapeutic agents, and chemical synthesis by-products. As a result, the publically available output of high throughput screening programs such as this will be useful in identifying more known anti-androgens in the environment. Nonetheless, WWTW effluent is a complex mixture of chemicals, their derivatives and transformation products, and further screening of chemicals without known activity will still be required.

The use of more advanced instrumentation can also reduce the number of unknown chemicals in complex samples to ease the identification of possible anti-androgens. For example, the use of gas chromatography coupled with time of flight mass spectrometry has a much higher mass accuracy than standard GC-MS and still allows for the use of spectral libraries (Jernberg *et al.*, 2013). Nonetheless, gas chromatography remains limited to nonpolar and medium polarity chemicals which can evaporate without

degradation (Burgess *et al.*, 2013). To avoid overlooking chemicals, alternative methods such as LC-MS/MS or Orbitrap could play an important role, particularly as accurate mass databases to identify specific chemicals advance (Becue *et al.*, 2011). Indeed, the Orbitrap has been recently employed in an EDA of anti-androgens in river sediment samples, identifying anti-androgenic organophosphates, musks, steroids and an oxygenated polyaromatic hydrocarbon (Weiss *et al.*, 2011).

Since anti-androgenic activity in the environment is associated with domestic WWTW effluent, environmental anti-androgens are also likely to be associated with human exposures (Johnson *et al.*, 2007b). This is well demonstrated by the detection of anti-androgenic activity in both domestic greywater and wastewater from Beijing, which was not completely removed during wastewater treatment (Ma *et al.*, 2013). Indeed, there is known human exposure to many of the identified and confirmed anti-androgens from this thesis. Some, including polyaromatic hydrocarbons, HHCB and triclosan have also been identified in human tissue, serum, urine and breast milk (Moon *et al.*, 2012; von der Ohe *et al.*, 2012; Reiner *et al.*, 2007; Somogyi and Beck, 1993). In fact, triclosan is now regularly monitored by the US Centres for Disease Control and Prevention as part of ongoing assessment of human exposure to chemicals (Centers for Disease Control and Prevention, 2013). Interestingly, the research presented in this thesis on anti-androgens in the environment has found itself in a similar situation to research on the implications of anti-androgenic EDCs on human health. Indeed, in a recent study, a combination of 22 AR antagonists at concentrations detected in human serum did not produce measurable effects in the MDA-kb2 cell line when tested in a mixture at concentrations at which they were detected in human serum (Kortenkamp *et al.*, 2014). Consequently, there also remains a knowledge gap between the known contaminants and the occurrence of reproductive abnormalities associated with testicular dysgenesis syndrome. This also suggests that additional chemicals with high potency anti-androgenic activity or high exposure are required to fill this gap. However, this study was based on AR antagonism and does not include alternative modes of anti-androgenic activity, such as steroidogenesis and prostaglandin synthesis inhibition, which may also contribute to the pathologies observed in the human population. This limits the results of the study since the human population is known to be exposed to some steroidogenesis inhibitors, such as the phthalate esters, which can cause cryptorchidism, hypospadias and impaired spermatogenesis in rodents (Sharpe and Skakkebaek, 2008).

Anti-androgenic chemicals with different mechanisms of action have been shown to act additively or even synergistically *in vivo* (Pottinger *et al.*, 2013; Hotchkiss *et al.*, 2010;

Christiansen *et al.*, 2009). In this thesis, by identifying anti-androgens by literature review, chemicals capable of inhibiting steroidogenesis were identified, such as 5α-reductase inhibiting fatty acids, which were generally inactive in the yeast screen. So far these mechanisms have not been considered as part of the multi-factorial aetiology of sexual disruption in the wild fish. Indeed, since 5α-dihydrotestosterone (DHT) was not previously thought to be a physiologically important androgen in fish, this mechanism of 5α-reductase inhibition has been largely ignored. However, recent research has detected DHT and mRNA for isoforms of the 5α-reductase enzyme in fish and found that the androgen can cause significant, masculinising affects *in vivo* (Margiotta-Casaluci *et al.*, 2013a; Martyniuk *et al.*, 2013; Margiotta-Casaluci and Sumpter, 2011). Furthermore, the pharmaceutical 5α-reductase inhibitor, dutasteride, caused reductions in fecundity and secondary sexual characteristics during a fathead minnows pair breeding assay (Margiotta-Casaluci *et al.*, 2013b).

Other steroidogenesis inhibitors have also been linked to effects associated with anti-androgens and include some pharmaceuticals. For example, clofibrate, gemfibrozil, ibuprofen, diclofenac, fluoxetine and fluvoxamide have been linked to the disruption of carp steroidogenic enzymes *in vitro* (Fernandes *et al.*, 2011). The non-steroidal anti-inflammatory inhibitor (NSAID), indomethacin, was also found to be anti-androgenic in both foetal rat and adult human testes explants, where testosterone production was reduced (Albert *et al.*, 2013; Kristensen *et al.*, 2012). However, the exact mechanism remains to be characterised. Recent research also found that maternal use of pharmaceutical analgesics, such as ibuprofen, asprin and paracetamol, was associated with cryptorchidism in newborns (Kristensen *et al.*, 2010). Interestingly though, this is likely to be a result of prostaglandin synthesis inhibition, rather than a direct effect on circulating androgens or AR antagonism. This is thought to occur through direct inhibition of cyclooxygenase (COX) enzymes, which mediate the conversion of archidonic acid to prostaglandins. Prostaglandins have been shown to play a role in male development, inducing Sertoli cell differentiation from somatic cells under the regulation of testosterone, demonstrating a role in the action of androgens (Adams and McLaren, 2002; Gupta, 1989). Consequently, prostaglandin synthesis inhibition may be an additional anti-androgenic mode of action for some EDCs (Kristensen *et al.*, 2011). Indeed, a number of known environmental EDCs, including benzophenones, phthalates and parabens, were found to be prostaglandin synthesis inhibitors in cell lines and *ex vivo* rat testes (Kristensen *et al.*, 2011). Nonetheless, there is little study of this mode of action in environmental samples, and this has not yet been considered within the multi-factorial aetiology to sexual disruption in fish. It also demonstrates that there are likely

to be other pharmaceuticals in the environment with the potential to cause androgen disruption other than just bicalutamide and cyproterone acetate. As a result, further study to identify and assess additional mechanisms of action is required to determine their potential implications for sexual disruption in fish, particularly during early development.

5.3 Assessing anti-androgenic pharmaceuticals *in vivo*

Research suggests that the steroid oestrogens, 17β-oestradiol (E2), oestrone (E1) and EE2 are the major drivers of sexual disruption in wild fish downstream of WWTW effluent outfalls (Tanna *et al.*, 2013; Maltret-Geraudie *et al.*, 2008; Jobling *et al.*, 2006; Allen *et al.*, 1999). This thesis provides further evidence to support this assertion, demonstrating that a mixture of the three steroid oestrogens at concentrations relevant to UK rivers and WWTW effluents could induce intersex in Japanese medaka in a dose responsive manner. However, whether this was an effect of a combination of the three oestrogens or that of a single chemical could not be proven by the experiment employed. Nonetheless, combined effects of oestrogenic chemicals have been reported *in vitro,* as well as in fish models for vitellogenin induction and reproductive endpoints (Brian *et al.*, 2007; Brian *et al.*, 2005; Rajapakse *et al.*, 2002; Silva *et al.*, 2002). It remains plausible that this will also be the case for intersex induction.

Predictive hydrological modelling supports the possibility of a multifactorial aetiology behind this condition involving androgen blockade by environmental AR antagonists (Jobling *et al.*, 2009). Associations between modelled concentrations of oestrogenic chemicals, oestrogenic and anti-androgenic activity at capture sites and the biological responses observed in fish (from Jobling *et al.*, 2006) were investigated. The data suggested that *"anti-androgens are strong causal factors [in the feminisation of wild fish in UK rivers], necessary for severe effects to occur"* (Jobling *et al.*, 2009). Indeed, predicted anti-androgenic activity at capture sites was positively correlated with vitellogenin induction, the presence of oocytes, intersex index and feminised ducts. There was also evidence of an additive effect of anti-androgenic activity in combination with steroid oestrogens influencing the presence of oocytes, intersex index and feminised ducts.

Whilst there is experimental evidence supporting the multi-factorial aetiology behind the induction of intersex in fish, there is little physical evidence to support the modelling study to suggest that this is occurring in the wild. Since anti-androgenic

pharmaceuticals were predicted to be present in the environment, the effects of bicalutamide and cyproterone acetate on sexual disruption at relevant concentrations were investigated in this project. This also incorporated exposures in combination with steroid oestrogens to further explore the possibility of a multi-factorial aetiology in sexual disruption. These experiments found no statistically significant effects of the chemicals on endpoints including vitellogenin induction, secondary sexual characteristics and intersex at their tested concentrations for the experimental durations and life stages tested. In combination with oestrogens, vitellogenin induction and intersex responses were slightly higher than the oestrogen treatment alone, but this was not statistically significant. Although cyproterone acetate has been shown to cause changes to plasma sex steroid levels in fish at these concentrations (Sharpe *et al.*, 2004), physiological responses, including intersex, have only been observed following exposure to concentrations in µg/L (Kiparissis *et al.*, 2003). Similarly for bicalutamide, intersex was not observed in an exposure of up to 100 µg/L (Panter *et al.*, 2012). As a result, the lack of significant effects observed during this thesis project is likely to be due to the comparatively low concentrations of anti-androgenic pharmaceuticals employed. This suggests that the presence of these drugs alone are unlikely pose an environmental threat to fish health. Nonetheless, these data do not refute experimental data indicating a multi-causal aetiology to sexual disruption. Nor do they refute the possibility that more complex mixtures of anti-androgenic activity in the environment could be a significant driver of sexual disruption alone.

Theoretically, a multi-factorial aetiology incorporating anti-androgens is well supported. Anti-androgenic chemicals can antagonise the AR to block androgen action at target sites and can also further disrupt the endogenous hormone balance by blocking the negative feedback of testosterone in the brain. This responds with increased release of gonadotrophic hormones, which in turn increases circulating concentrations of steroid hormones to produce an oestrogenic internal environment due to the continued blockade of the AR (Jensen *et al.*, 2004; O'Connor *et al.*, 2002; Staiman and Lowe, 1997). Consequently, previous research has found that anti-androgenic chemicals cause demasculinisation in fish, including reductions in secondary sexual characteristics, effects on behaviour, intersex induction and low levels of vitellogenin induction (Sebire *et al.*, 2009; Sebire *et al.*, 2008; Kang *et al.*, 2006; Ankley *et al.*, 2004; Panter *et al.*, 2004). In addition, at a molecular level, anti-androgens have been shown to decrease the expression of multiple genes involved in testicular differentiation (Rajakumar *et al.*, 2012; Filby *et al.*, 2007a). However, although there is abundant evidence to suggest that anti-androgens can act additively (Pottinger *et al.*, 2013;

Hotchkiss *et al.*, 2010; Christiansen *et al.*, 2009; Hass *et al.*, 2007; Earl Gray Jr. *et al.*, 2006), apart from the experiments described in this thesis there has been little attention paid to the possibility of interactions between anti-androgens and oestrogens. There is one report of a set of bioavailable anti-androgens enhancing the incidence of ovarian cavities in roach in combination with 3 ng/L EE2 (Lange *et al.*, 2012a). However, this has not yet been published in full. Nonetheless, mechanistically the possibility of combined effects seems plausible. Indeed, an increase in the internal oestrogenic environment by anti-androgens through blockade of the AR or feedback on sex steroids could be supplemented by exposure to environmental oestrogens. In addition, flutamide has been shown to increase the expression of oestrogen receptors β and γ in fathead minnows (Filby *et al.*, 2007b) and oestrogen receptor α in a recent study with Murray rainbowfish (Bhatia *et al.*, 2014). This may suggest that oestrogens could act additively with anti-androgens in a mixture context, possibly with anti-androgens sensitising an organism to oestrogenic effects. This could be assessed through mechanistic studies with fish, for example by exposing fish to flutamide and E2 alone and in combination at a range of doses. This would determine whether androgen blockade in conjunction with oestrogen exposure could induce intersex in a mixture scenario. However, increasing appreciation of anti-androgenic chemicals that operate without interacting with the AR highlights the possibility that additional modes of action may be involved in the multifactorial aetiology to sexual disruption. Indeed, endocrine activity of WWTW effluents and their constituent chemicals is not limited to only anti-androgenic and oestrogenic activity. This has been recently highlighted by an assessment of 10 Australian water samples, including WWTW effluent, surface water and recycled water, by 103 unique bioassays for a range of biological endpoints (Escher *et al.*, 2014). This found that 63% of the assays were active in at least one water sample. The most responsive biological endpoints related to xenobiotic metabolism, genotoxicity, hormone mediated modes of action and the adaptive stress response pathway. As a result, to truly determine whether chemicals in effluent other than the steroid oestrogens can cause or contribute to sexual disruption, exposure of fish to fractionated WWTW effluent could be employed. This could involve exposure to a mixture of the non-oestrogenic fractions alone and in combination with steroid oestrogens, as shown in Figure 5.2.

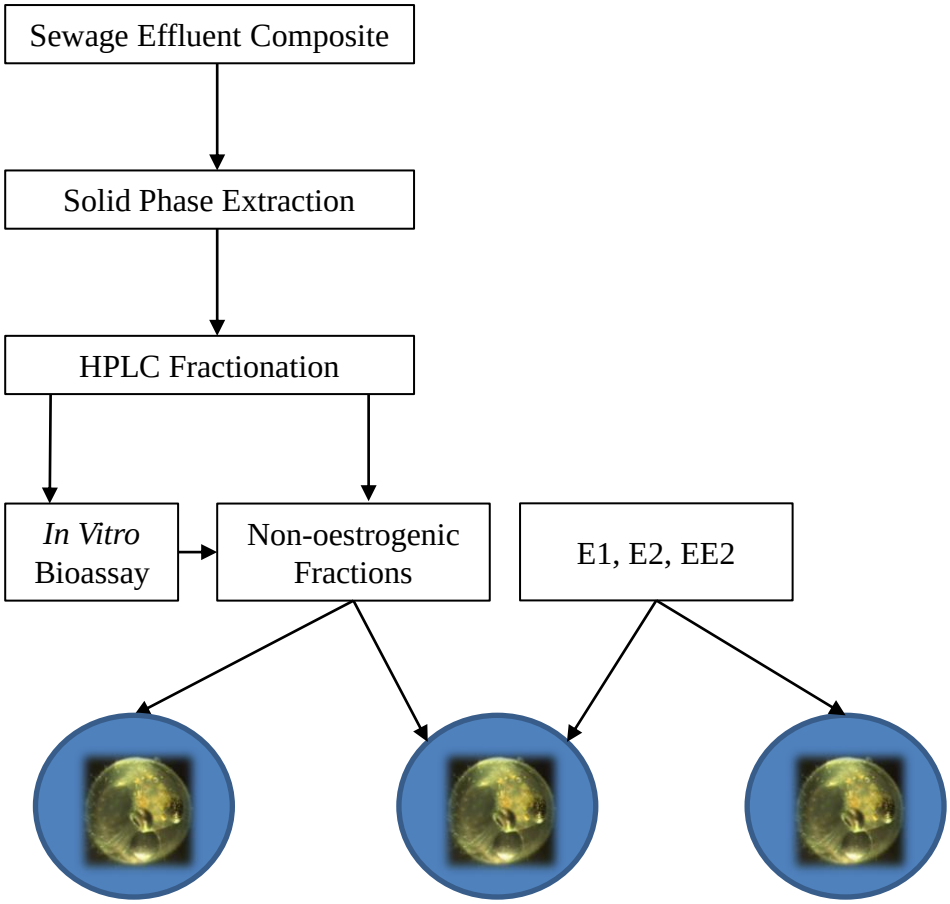


Figure 5.2 *Experimental design for exposure of Japanese medaka embryos to WWTW effluent fractions to assess the multi-factorial aetiology of sexual disruption.*

This could also incorporate more modern molecular methods for assessing the intersex condition using biomarkers, which have been developed in medaka (Hirakawa *et al.*, 2012; Zhao and Hu, 2012; Flynn *et al.*, 2010). A number of oocyte genes have been proposed as biomarkers, of which one of the most applicable is ovary structure protein 1 (*osp1*). Out of a set of candidate genes, *osp1* achieved the highest transcriptional induction in males following EE2 exposure, which was comparable to that of unexposed females. In comparison to histological processing, the gene expression correlated well with intersex severity and it was expressed in a dose responsive manner to EE2 exposure (Zhao and Hu, 2012). More recently, *osp1* has been linked to green fluorescent protein (GFP) in transgenic medaka in a newly developed assay. This induced intersex following a 90 day exposure to 0.75 ng/L EE2, where individual oocytes could be identified in the testes and quantified by fluorescence (Zhao *et al.*, 2014). This response can also be used in combination with the male sex determining *dmy* gene or secondary sexual characteristics to identify intersex individuals and to

quantify severity, avoiding time consuming histological processing. It has also proven to be more sensitive than histology, which can produce false negatives due to the limited observation of the total gonad area and the difficulties in differentiating the early stages of oocyte development from spermatogonia (Zhao *et al.*, 2014). Nonetheless, it does not provide as much information as histology, which can identify any other abnormal physiology (such as fibrosis) in addition to the intersex endpoint. The GFP induction observed in the testis of fish exposed to EE2 by Zhou *et al.* is displayed below in Figure 5.3.

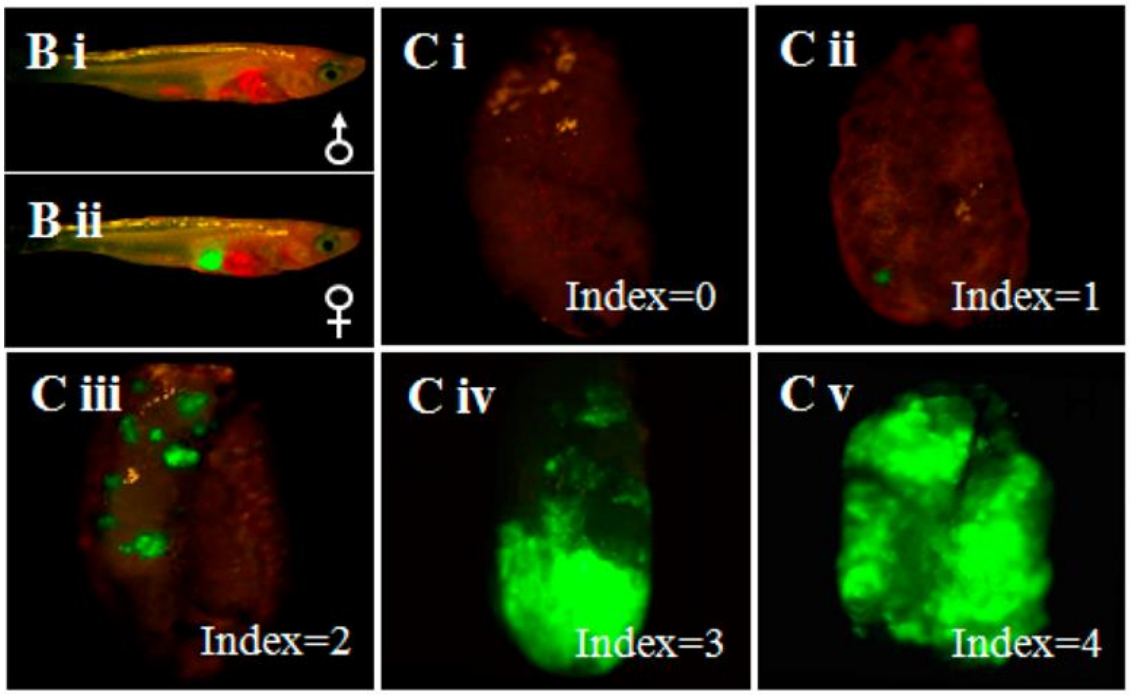


Figure 5.3 *Fluorescent microscopy observing the GFP response, linked to the oocyte gene osp1, in the excised testes of transgenic Japanese medaka following exposure to increasing doses of EE2* (from Zhao *et al.*, 2014)*. The intersex index is also indicated.*

5.4 Future Trends

This thesis expanded on the aim of identifying and assessing environmental anti-androgens by analysing future trends in environmental concentrations. This is in fact the first analysis of future trends in concentrations of anthropogenic environmental contaminants and the first incidence of predictive modelling being adapted for use in Australia to assess endocrine disruptors. Predictions found that increasing population pressure on WWTWs could increase the concentrations of human derived contaminants entering the environment in WWTW effluent in UK and South Australian

catchments by 2050. Concurrently, decreasing effluent dilution caused by a climate induced reduction in river flow was expected to further increase the concentrations of these contaminants in receiving waters. Based on a medium level emissions scenario and assuming that current trends for demographic assumptions of fertility, mortality and migration would prevail in the future (Projection B), environmental concentrations were expected to double. This work focussed on the steroid oestrogens and anti-androgenic pharmaceuticals in two catchments only. However, work is underway at the Centre for Ecology and Hydrology, Wallingford to expand the hydrological modelling of steroid oestrogens under population and climate projections for 2050 to river catchments throughout England and Wales. The results so far have been agreeable with those described here, predicting modest increases in steroid oestrogen concentrations. Indeed, when compared to the maps produced in 2009 (Williams *et al.*, 2009), the number of stretches at risk of endocrine disruption were expected to moderately increase by 5-7% dependant on the climate projection, whilst stretches at high risk increased 2-3% (Keller, 2014 personal communication).

Increasing concentrations of steroid oestrogens in the future indicate that their presence in the environment will be a long term issue, unless mitigating action is taken. Due to the natural human origin of E1 and E2 and the importance of the pharmaceutical EE2 within society, mitigating these chemicals at source is not possible. Instead, improvements in their removal during the wastewater treatment process are required. From 2005-10 the UK's Endocrine Disruption Demonstration Program trialled advanced treatment processes, including adsorption onto granular activated carbon (GAC) and advanced chemical oxidation with ozone or chlorination. These were found to satisfy predicted no effect concentrations (PNECs) for E1 and E2 (1 and 3 ng/L), whilst the PNEC for EE2 (0.1 ng/L) could be satisfied with limited dilution (Butwell *et al.*, 2010). New innovations in wastewater treatment are also being assessed and considered for future use, dependant on their ability to remove a range of chemicals and their ease of integration into WWTWs, as well as their economic costs and carbon footprint. These include the use of ultrasound to enhance degradation by UV photo oxidation and ozone, as well as advances in carbon products for adsorption, similar to granular activated carbon (Dee *et al.*, 2013). In addition, there has been increasing interest in the use of catalysts, such as Fe-TAML, which triggers oxidation, to break down these chemicals. Indeed, preliminary data from the use of Fe-TAML proves highly promising. It was able to rapidly degrade the steroid oestrogens, with a half-life of around five minutes, causing a 95% loss in around 15 minutes and a concomitant loss of estrogenic activity *in vitro* (Shappell *et al.*, 2008; Brown, 2006).

This suggests that it is not producing any active oestrogenic by-products, which have been observed during other treatment processes (Moriyama *et al.*, 2004).

In 2012, the European Commission proposed to add E2 and EE2 to a list of "priority substances" under the Water Framework Directive. This European Union Directive was set up in 2000 to provide measures against chemical pollution of surface waters by regulating priority substances, which are monitored and controlled by member states based on environmental quality standards (EQSs). However, the pharmaceutical and water industries lobbied heavily against the proposal and cited insufficient evidence for population level effects of sexual disruption (McKie, 2012). In part this was a response to the associated cost of upgrading WWTWs to remove these chemicals, which in England and Wales was estimated at £30 billion, causing controversy regarding who should bear the cost (Owen and Jobling, 2012). Eventually, E2 and EE2 were instead adopted under a "watch list" to gather monitoring data to facilitate the determination of appropriate measures to address the risk that they pose (European Parliament, 2013). They therefore have the potential to be added to the priority substances list with defined EQSs in 2016.

This thesis provides further evidence for the environmental role of steroid oestrogens on sexual disruption. In addition, the projected increase in environmental concentrations, supported by the extension of this work by the Centre for Ecology and Hydrology, suggests that the risk posed to fish by steroid oestrogens is likely to increase in the UK in the future. Consequently, from the UK perspective, this research supports the addition of E2 and EE2 to the list of priority substances and the requirement for mitigation. To broaden this research, the integration of predictive hydrological modelling with climate and population change scenarios could be used to assess changes to the risk they pose in other European river catchments in the future. If the conclusions are similar to the UK, this will provide additional evidence to support regulatory action. However, a recent study found that roach populations in effluent contaminated river stretches in Southern England were self-sustaining, despite the presence of steroid oestrogens and the occurrence of intersex (Hamilton *et al.*, 2014). As a result, in the absence of clear population level effects in the wild, regulatory action would require the political will to consider sexual disruption as environmental damage to be rectified. Alternatively, the precautionary principle enshrined within the European Commission's Action Program for 2020 (European Commission, 2014) would need to be acknowledged to legislate on the grounds of avoiding future population level damage.

The level of increase in the predicted environmental concentrations of these contaminants may be a good indicator of the change in concentrations of other anthropogenic pollutants. Reductions in available dilution and/or increasing input are likely to increase the concentrations of a variety of contaminants, including some priority pollutants, such as polyaromatic hydrocarbons. As a result, meeting the regulatory requirement of the EQSs may become more difficult without mitigation. Furthermore, increasing the concentrations of other chemicals, which are not considered as priority pollutants, may produce greater additional risks to fish populations. This may be particularly relevant to some pharmaceuticals, which have been shown to cause adverse effects in fish at concentrations close to those detected in the environment. For example, recent research on synthetic progestagens, which are combined with EE2 in the contraceptive pill to inhibit ovulation, has also found that these drugs can effect reproduction in fish at low concentrations. Indeed, levonorgestrol and gestodene have been shown to decrease egg production in fathead minnows at 0.8 and 1 ng/L respectively in two separate studies (Runnalls *et al.*, 2013; Zeilinger *et al.*, 2009). Interaction with the AR also occurred to cause masculinisation of the secondary sexual characteristics of females. In addition, norethindrone reduced egg production in fathead minnows and medaka at 1.2 and 25 ng/L, respectively. There is little data available on the environmental presence of progestagens, but they are expected to occur in rivers in high pg/L to low ng/L levels (Runnalls *et al.*, 2013), potentially within the range of their *in vivo* effects. Indeed, at present norethindrone has a similar annual use to bicalutamide and cyproterone acetate and could therefore occur at concentrations similar to those predicted for the anti-androgens during this project. Another set of pharmaceuticals of interest are the NSAIDs, of which naproxen, ibuprofen, diclofenac and an indomethacin metabolite were detected in WWTW effluents, as described in chapter four. These are highly consumed, making up around 7% of all prescriptions in Europe, and are commonly used by elderly people for pain management. In fact, an epidemiology study from the US reported that 70% of people over 65 used NSAIDs at least once weekly (Pilotto *et al.*, 2010). Consequently, their use in the future could further increase due to the aging population. As a result of their widespread use, some NSAIDs have been detected at low µg/L concentrations in the aquatic environment and identified in the bile of wild fish downstream of effluent discharges (Brozinski *et al.*, 2013). Since they inhibit prostaglandin synthesis there is some cause for concern for effects on fish health. Indeed, one study found spawning frequency was reduced in medaka at low µg/L concentrations of ibuprofen (Flippin *et al.*, 2007). However, this was not replicated in a second study at higher concentrations, which found no effect on reproduction in zebrafish, although endogenous prostaglandin

was reduced (Morthorst *et al.*, 2013). There has also been data published on effects of diclofenac on the kidney and intestinal structure of fish at environmentally relevant concentrations (e.g. Mehinto *et al.*, 2010). However, when histology from studies reporting effects of diclofenac at low concentrations were reassessed by a panel of pathologists, no adverse effects were found below a proposed NOEC of 320 µg/L, based on data from two early life stage tests in rainbow trout and zebrafish (Wolf *et al.*, 2014; Memmert *et al.*, 2013). Nonetheless, diclofenac was added to the watch list by the European Commission under the Water Framework Directive in 2012, alongside E2 and EE2.

In contrast to the UK and Europe, in Australia there are no regulations or EQSs in place for many of the Water Framework Directive's priority pollutants or the steroid oestrogens. Predictive modelling indicates that steroid oestrogen concentrations in WWTW effluents in South Australia and discharge in a modelled downstream river stretch could put fish at risk of endocrine disruptive effects. This supports analytical measurements of steroid oestrogens in WWTW effluents from across Australia, which detected them at similar concentrations (Allinson *et al.*, 2010; Mispagel *et al.*, 2009; Ying *et al.*, 2009; Ying *et al.*, 2008; Tan *et al.*, 2007; Williams *et al.*, 2007; Braga *et al.*, 2005; Li *et al.*, 2004). The river concentrations also compare well with those measured at five river sites in Queensland at effluent outfalls and 1 km downstream of WWTWs, which also indicated a risk of endocrine disruption occurring (Ying *et al.*, 2009). In light of the widespread presence of steroid oestrogens in Australian effluents, their detection in rivers and their projected increase in concentration in the future, there is a requirement for a greater understanding of their effects on wild, native fish species. Increasing research has demonstrated a susceptibility of some native species to steroid oestrogens, such as barramundi, black bream and Murray rainbowfish (Woods and Kumar, 2011; Codi King *et al.*, 2008). However, assessments of the effects of whole effluent exposures and studies of wild fish have so far found little evidence of a health threat to fish. Indeed, in 2012 the Commonwealth Scientific and Industrial Research Organisation (CSIRO) published an impact assessment of treated effluent in the aquatic environment. This involved exposures of fish under laboratory conditions and *in situ* at WWTWs using a mobile fish laboratory, as well as an assessment of wild, invasive common carp in the River Yarra, Victoria (Kumar *et al.*, 2012). This study found no evidence of oestrogenic effects, such as vitellogenin or intersex induction in either zebrafish or Murray rainbowfish exposed to 100% WWTW effluent for 28 days. In addition, no incidences of vitellogenin induction or gonadal abnormalities were detected in carp caught on the River Yarra. As a result, it has been suggested that the risk to

fish health indicated by the modelling study in this thesis should be *"treated with caution pending further research"* (Sutcliffe *et al.*, 2013). However, it should be considered that the WWTW effluents employed in fish exposures by the CSIRO study were all tertiary treated and the steroid oestrogens, E2 and EE2, were not detected during the studies, whilst E1 was detected infrequently. The WWTWs discharging into the River Yarra were also tertiary treated and no oestrogenic activity was detected at the carp capture sites. Without the steroid oestrogens present, it is unsurprising that no oestrogenic effects were observed. Consequently, there is a requirement for further study of the health effects of WWTW effluent on Australian fish species to examine the conclusions of the modelling work. However, this should include assessments of the effects of exposing fish to known oestrogenic effluents from WWTWs which do not use advanced treatment technologies to analyse worst case scenarios. For assessing intersex induction this should also utilise early life studies to ensure that critical stages of development are included in the exposure. This thesis has shown that Japanese medaka are a highly suitable test species for such a study. However, increasing research with the Murray rainbowfish suggests that they have a comparable sensitivity to oestrogenic disruption and therefore make a suitable native test species (Woods and Kumar, 2011). However, to the author's knowledge, there is no evidence of intersex being induced in this species during *in vivo* exposures and so this requires further development. In terms of field assessments, the capture of wild fish for the assessment of reproductive health effects should be focussed on at risk or high risk areas, based on low dilution and lower level treatment processes upstream. From this aspect, hydrological modelling could be a useful first line tool in identifying oestrogenic WWTW effluents and at risk areas in Australian river catchments to guide field assessments.

Whilst Australian fish species may be considered at risk of endocrine disruption, until more direct evidence arises of effects of EDCs in wild species, there is unlikely to be any perceived requirement or move towards mitigation. However, increasing interest in sustainable wastewater reuse may provide a new impetus towards mitigating potentially rising concentrations of EDCs in effluents. Indeed, the interest in using reclaimed water for agriculture and supplementing the drinking water supplies will make costs associated with mitigation unavoidable to ensure that human health is not affected (Falconer *et al.*, 2006). Furthermore, the indigenous communities of Australia have strong sensitivities towards water quality, which is causing greater public awareness in water quality management and may also influence the likelihood of mitigation in the future (Kookana *et al.*, 2013).

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APPENDIX 1

A simple worked example of effluent modelling at a South Australian WWTW

Below is a simple, fictitious worked example of the calculations employed in this study to estimate effluent concentrations in the UK and South Australia from cohort data. Please note that the calculations below should be taken as a guideline as they are worked by hand and will therefore differ from the more extensive calculations performed in MS Excel for this project.

Creating Cohorts

Census data
Females age 15-50: 300
Females age ≥ 51: 200
Males age ≥ 15: 500
Males and Females age 0: 50
7.6% of females over 50 using HRT

Cohort Numbers:
Pregnant females = 50 (based on males and females aged 0)
Menstrual females = 300 – 50 = 250
Menopausal females on HRT = 200 × 0.076 = 15
Menopausal females = 200 – 15 = 185
Males = 500
TOTAL POPULATION: 1000

Cohort Percentages:
Pregnant females = 25.0%
Menstrual females = 18.5%
Menopausal females on HRT = 1.5%
Menopausal females = 5.0%
Males = 50.0%

Calculating the per capita consumption of E1 and E2

$$SE2 = 0.5 \sum_{i=1}^{n} fi\ (UE2)$$

$$SE1 = \sum_{i=1}^{n} fi\ (UE1) + 0.5\, SE2$$

Where *S* is the per capita consumption of oestrogen arriving at a WWTW (µg/d), *n* is the number of cohorts and *U* is the total oestrogen excreted in urine (in free, glucuronide and sulphate forms) and faeces for each cohort fraction (*fi*) of the population. A KT value for E2 of 0.5 is incorporated assuming that 50% will be degraded to E1 in transit in the sewerage system.

Cohort	Criteria	Number of people in 2008 Census Australia	% of population Australia	Mean excretion per person (µg/d)	
				E2	E1
Menstrual females	Age 15-50 (minus pregnant women)	4797348	24.2%	3.2	11.7
Menopausal females	Age >51 (minus menopausal women on HRT)	2718963	13.7%	1.0	1.8
Menopausal females on HRT	7.6% UK and 11.8% Australian menopausal females (>51)	363761	1.8%	56.1	28.4
Pregnant Females	1/22 UK and 1/19 Australian menstrual females	260101	1.3%	393	550
Males	Age 15-50	7777909	39.2%	1.8	2.6
	TOTAL	15918082			

$SE2 = 0.5 \times ((0.242 \times 3.2) + (0.137 \times 1) + (0.018 \times 56.1) + (0.013 \times 393) + (0.392 \times 1.8))$
$SE2 = 0.5 \times (0.78 + 0.14 + 1.01 + 5.11 + 0.71)$
$SE2 = 0.5 \times 97.75$
$\underline{SE2 = 3.9\ \mu g/d}$

$SE1 = ((0.242 \times 11.7) + (0.137 \times 1.8) + (0.018 \times 28.4) \times (0.013 \times 550) \times (0.392 \times 2.6)) + 3.9$
$SE1 = (2.8 + 0.25 + 0.51 + 7.15 + 1.02) + 3.9$
$SE1 = 11.73 + 3.9$
$\underline{SE1 = 15.63\ \mu g/d}$

Calculating the per capita consumption of EE2

Total active compound prescribed (2008) = 5.5kg
There were 366 days in 2008
Estimated Population of Australia = 22000000
Excretion rate = 40%

EE2 consumption per day = 5.5 ÷ 366 = 0.015kg
EE2 excreted per person = (0.015 × 0.4) ÷ 22000000
$SEE2 = 2.72 \times 10^{-10}$(kg/d)
$\underline{SEE2 = 0.27\ \mu g/d}$

Calculating Effluent Concentrations using E2 as an example

WWTW Data
Population served = 2761
Domestic Flow = 230000 L/day
Non Domestic Flow = 50000 L/day
Removal rates (E1, E2, EE2) = 64.7%, 81.7%, 85.2%

Influent Concentration (E2) = (3.9 × 2761) ÷ 230000 = 0.047µg/L = **47ng/L**

Effluent Concentration (E2) = ((3.9 × 2761) ÷ 280000) × (1-0.817) × 1000 = **7.02 ng/L**

APPENDIX 2

Silylisation Protocol

1. Sample bottles must be clean and dry. If necessary, solvent rinse and evaporate off remaining solvent before and heating bottles in a drying oven to ensure this.

2. After cooling, fill the clean and dry sample bottles with a small volume of the sylilating reagent, Sylon CT (5% dimethyldichlorosilane in toluene from Sigma Aldrich, UK). Close the sample bottle and shake to ensure that all the walls are covered by the reagent. Leave for 30 minutes and shake periodically.

3. Pour the excess of Sylon TC into a collecting vessel for reuse.

4. Rinse the sample bottle containing the residue Sylon TC twice with a small volume of toluene and two times with methanol (dispose of waste toluene and waste methanol in chlorinated waste).

5. Finally dry the bottles for three hours at 105^{o}C.

Tank conditions from experiment one

Table A2.1 *The temperature range in treatment tanks during experiment one*

	Temperature (^{o}C)			
	Mean	Min	Max	Range
Control A	25.3	24.9	26.2	1.3
Control B	25.2	24.4	26.1	1.7
Solvent Control A	25.5	25.1	26.2	1.1
Solvent Control B	25.4	24.5	26.2	1.7
Anti-Androgen A	25.6	25.2	26.3	1.1
Anti-Androgen B	25.5	25	26.3	1.3
Oestrogen A	25.6	25.1	26.4	1.3
Oestrogen B	25.5	24.9	26.4	1.5
Mixture A	25.7	25.2	26.4	1.2
Mixture B	25.5	24.9	26.4	1.5
Positive Control A	25.6	25.3	26.3	1
Positive Control B	25.4	25	26.3	1.3

Table A2.2 *The percentage air saturation in treatment tanks during experiment two, based on the measured dissolved oxygen.*

	Dissolved Oxygen: % Air Saturation			
	Mean	Min	Max	Range
Control A	104%	98%	111%	13%
Control B	102%	96%	111%	15%
Solvent Control A	101%	92%	111%	19%
Solvent Control B	101%	96%	111%	14%
Anti-Androgen A	99%	87%	113%	26%
Anti-Androgen B	100%	93%	110%	17%
Oestrogen A	101%	94%	112%	18%
Oestrogen B	101%	96%	110%	14%
Mixture A	102%	95%	113%	19%
Mixture B	101%	98%	110%	13%
Positive Control A	101%	95%	113%	18%
Positive Control B	100%	95%	109%	15%

Tank conditions from experiment two

Table A2.4 *The temperature range in treatment tanks during experiment two. This was the highest in control tank A where mortality occurred.*

	Temperature (^{o}C)			
	Mean	Min	Max	Range
Control A	25.8	22.1	27.8	5.7
Control B	26.2	24.9	26.8	1.9
Solvent Control A	26.0	22.9	26.7	3.8
Solvent Control B	26.0	25.6	26.5	0.9
Low Anti-Androgen A	25.9	22.8	26.5	3.7
Low Anti-Androgen B	26.0	25.2	26.5	1.3
Low Oestrogen A	26.1	22.5	26.7	4.2
Low Oestrogen B	26.0	24.9	26.5	1.6
Low Mixture A	26.2	22.7	26.8	4.1
Low Mixture B	26.0	25.0	26.5	1.5
High Oestrogen A	26.2	22.9	26.7	3.8
High Oestrogen B	25.9	24.9	26.5	1.6
High Anti-Androgen A	26.1	22.6	26.8	4.2
High Anti-Androgen B	25.9	24.9	26.5	1.6
High Mixture A	26.1	22.7	26.8	4.1
High Mixture B	25.9	24.5	26.4	1.9
Positive Control A	26.0	23.2	26.9	3.7
Positive Control B	25.8	25.1	26.4	1.3

Table A2.5 *The percentage air saturation in treatment tanks during experiment two, based on the measured dissolved oxygen.*

	Dissolved Oxygen: % Air Saturation			
	Mean	Min	Max	Range
Control A	100%	95%	106%	11%
Control B	100%	97%	104%	7%
Solvent Control A	92%	70%	102%	32%
Solvent Control B	91%	78%	126%	48%
Low Anti-Androgen A	89%	74%	100%	26%
Low Anti-Androgen B	89%	77%	109%	32%
Low Oestrogen A	87%	68%	101%	33%
Low Oestrogen B	89%	71%	100%	30%
Low Mixture A	89%	75%	100%	24%
Low Mixture B	90%	78%	101%	23%
High Oestrogen A	88%	73%	99%	26%
High Oestrogen B	90%	75%	100%	26%
High Anti-Androgen A	89%	75%	100%	25%
High Anti-Androgen B	90%	73%	101%	27%
High Mixture A	89%	71%	101%	30%
High Mixture B	89%	72%	108%	35%
Positive Control A	91%	79%	100%	21%
Positive Control B	90%	75%	100%	25%

Table A2.6 *The measured nitrate, nitrite and ammonia concentrations measured using API aquarium test strips (API, UK)*

	Nitrates (mg/L)			Nitrites (mg/L)			Ammonia (mg/L)			pH		
	mean	min	max	mean	min	max	mean	min	max	mean	min	max
Control A	12	0	40	0.2	0.0	0.5	0.1	0.0	0.5	7.7	7.3	8.2
Control B	13	0	40	0.1	0.0	0.5	0.1	0.0	0.5	7.7	7.6	7.9
Solvent Control A	13	0	40	0.2	0.0	0.5	0.2	0.0	1.0	7.7	7.5	7.9
Solvent Control B	13	0	40	0.2	0.0	0.5	0.2	0.0	1.0	7.7	7.6	7.9
Low Anti-Androgen A	13	0	40	0.2	0.0	0.5	0.2	0.0	1.0	7.8	7.5	8.0
Low Anti-Androgen B	14	0	40	0.1	0.0	0.5	0.2	0.0	1.0	7.7	7.5	7.9
High Anti-Androgen A	13	0	40	0.2	0.0	0.5	0.1	0.0	1.0	7.7	7.4	7.9
High Anti-Androgen B	13	0	40	0.1	0.0	0.5	0.2	0.0	1.0	7.7	7.7	7.9
Low Oestrogen A	13	0	40	0.2	0.0	0.5	0.1	0.0	0.5	7.7	7.4	7.9
Low Oestrogen B	13	0	40	0.2	0.0	0.5	0.1	0.0	0.5	7.7	7.7	8.0
High Oestrogen A	13	0	40	0.1	0.0	0.5	0.1	0.0	1.0	7.7	7.3	7.9
High Oestrogen B	13	0	40	0.2	0.0	0.5	0.1	0.0	0.5	7.8	7.7	8.0
Low Mixture A	13	0	40	0.1	0.0	0.5	0.1	0.0	0.5	7.7	7.5	7.9
Low Mixture B	14	0	40	0.1	0.0	0.5	0.1	0.0	1.0	7.7	7.7	7.9
High Mixture A	13	0	40	0.2	0.0	0.5	0.1	0.0	0.5	7.7	7.5	8.0
High Mixture B	13	0	40	0.2	0.0	0.5	0.1	0.0	1.0	7.8	7.6	8.1
Positive Control A	14	0	40	0.2	0.0	0.5	0.1	0.0	0.5	7.7	7.5	7.9
Positive Control B	14	0	40	0.2	0.0	0.5	0.1	0.0	1.0	7.7	7.7	7.9

Severe fibrosis in an intersex gonad

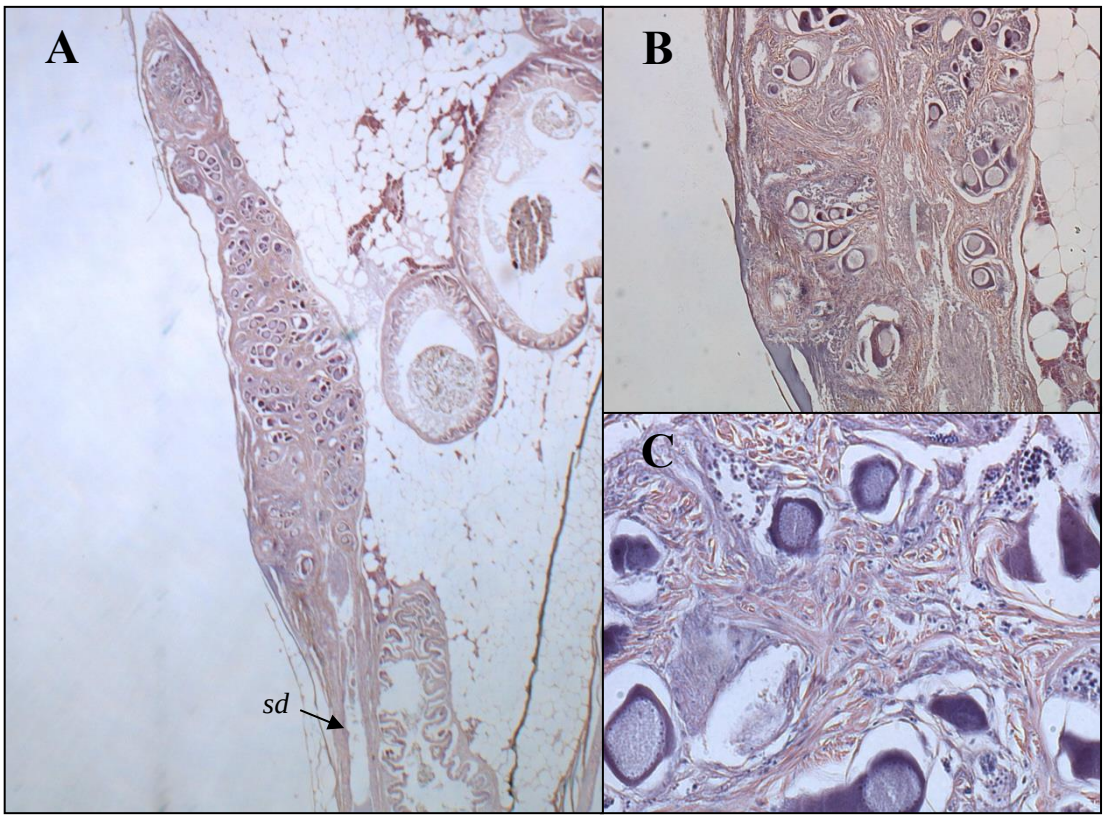

Figure A2.1 *A severely fibrotic gonad with sporadic oocytes. Image (A) was taken at x20 magnification, (B) at 100x and (C) at 400x. The gonad has a similar shape to the testis of male fish, albeit smaller, and some testicular tissue was observed alongside primary oocytes indicating a feminised male. Since oocytes and fibrotic tissue made up the majority of the testes and the sperm duct (sd) was empty, this fish was considered to be severely feminised with an index of 6.*

APPENDIX 3

A3.1 Solid phase extraction

Solid phase extraction (SPE) is an important step in sample preparation for analytical chemistry procedures. It is a chromatography method in which analytes in a liquid phase are passed through a column containing a solid phase where selective partitioning occurs to isolate analytes from the solution. Consequently, SPE can be used to “clean up” samples by removing unwanted chemicals or to concentrate and enrich samples by capturing analytes present at low concentrations from the liquid phase. Specifically, when an aqueous liquid phase is passed through the solid phase, its chemical constituents can be captured by retention to the sorbent material within the cartridge. The solid phase is then dried to remove the aqueous phase and a low volume of solvent is passed through which disrupts the interaction of the chemicals with the sorbent and elutes them. SPE is a very versatile technique and can be adapted to the needs of the researcher to capture a narrow set of chemicals or a broader range depending on the solid phase, solvents and conditions such as pH (Koester and Moulik, 2005).

A3.2 Fractionation by high performance liquid chromatography

Like SPE, high performance liquid chromatography (HPLC) is a chromatography method in which a mobile phase is delivered at high pressure through a column containing a stationary phase of sorbent material similar to that used in SPE. As the mobile phase moves through the stationary phase, different chemicals have different affinities for each phase dependant on their polarity. Consequently they move through the column with the mobile phase at different speeds, with those with a high affinity to the stationary phase moving the slowest. This allows them to be separated based on their retention time within the column. Gradient elutions with an elutrophic series of changing solvent concentrations can be used in the mobile phase to separate chemicals with a wide range of polarity. This is particularly important in EDA for maximising the recovery and separation of chemicals from the environmental samples in a shorter time period, ensuring that the most non-polar chemicals are eluted.

A3.3 Yeast androgen screen

The recombinant yeast androgen screen (YAS) was developed by Glaxo (now GlaxoSmithKline) and is a strain (PGKhAR) of *Saccharomyces cerevisiae* stably transfected with the gene coding for the human androgen receptor (hAR). This is linked to a reporter gene, *lacZ*, through an androgen response element which is capable of interacting with the receptor. LacZ codes for the enzyme β-galactosidase, which enters the medium in which the cells are maintained and metabolises the chromogenic substrate chlorophenol red β-galactopyranoside (CPRG) to produce a yellow to red colour change. This response can then be measured by absorbance at 540 nm. Consequently, dosing the cells with an androgen will cause receptor binding, enzyme production and a colour change in a dose dependant manner, which can be used to quantify the activity of a chemical. Whilst this can be used to assess androgens, anti-androgenic activity can also be quantified by dosing the cells with a concentration of a natural AR agonistic ligand, such as DHT, capable of inducing at a submaximal response (~65%). If this is dosed in combination with an anti-androgen, inhibition of the colour change through androgen receptor antagonism can be used to assess activity (Sohoni and Sumpter, 1998; Purvis *et al.*, 1991).

A3.4 Testing fractions in the yeast screen: Raw data

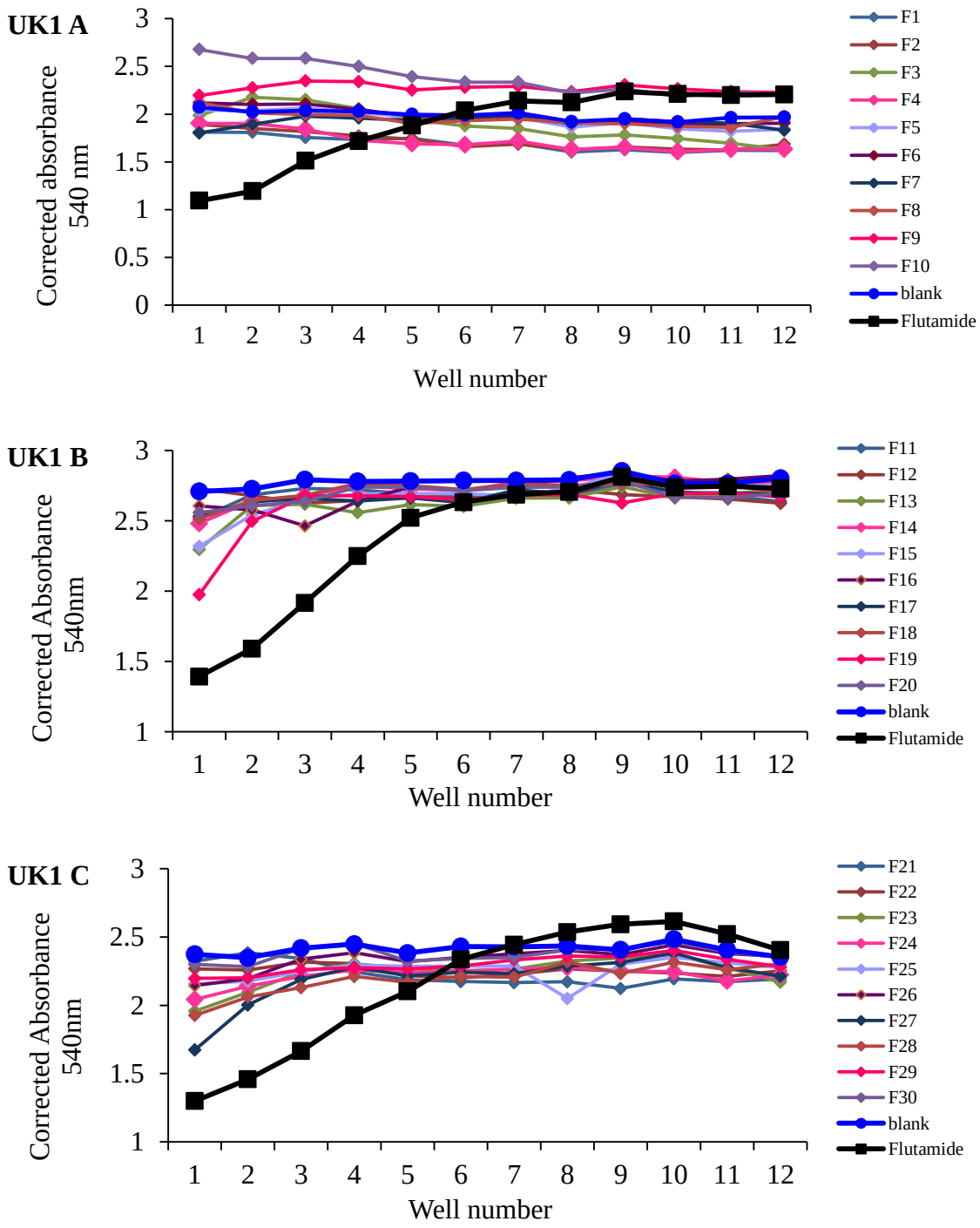

Figure A3.1 *Results from three yeast androgen screens (A,B &C) assessing fractions from UK1 with a DHT raised background (2 nM) to detect anti-androgenic activity. Flutamide was used as a positive control at concentrations serially diluted from 50 nM (well 1) to 0.02 nM (well 12). Fractions were serially diluted from wells 2 to 12, with well 1 containing the pure fraction.*

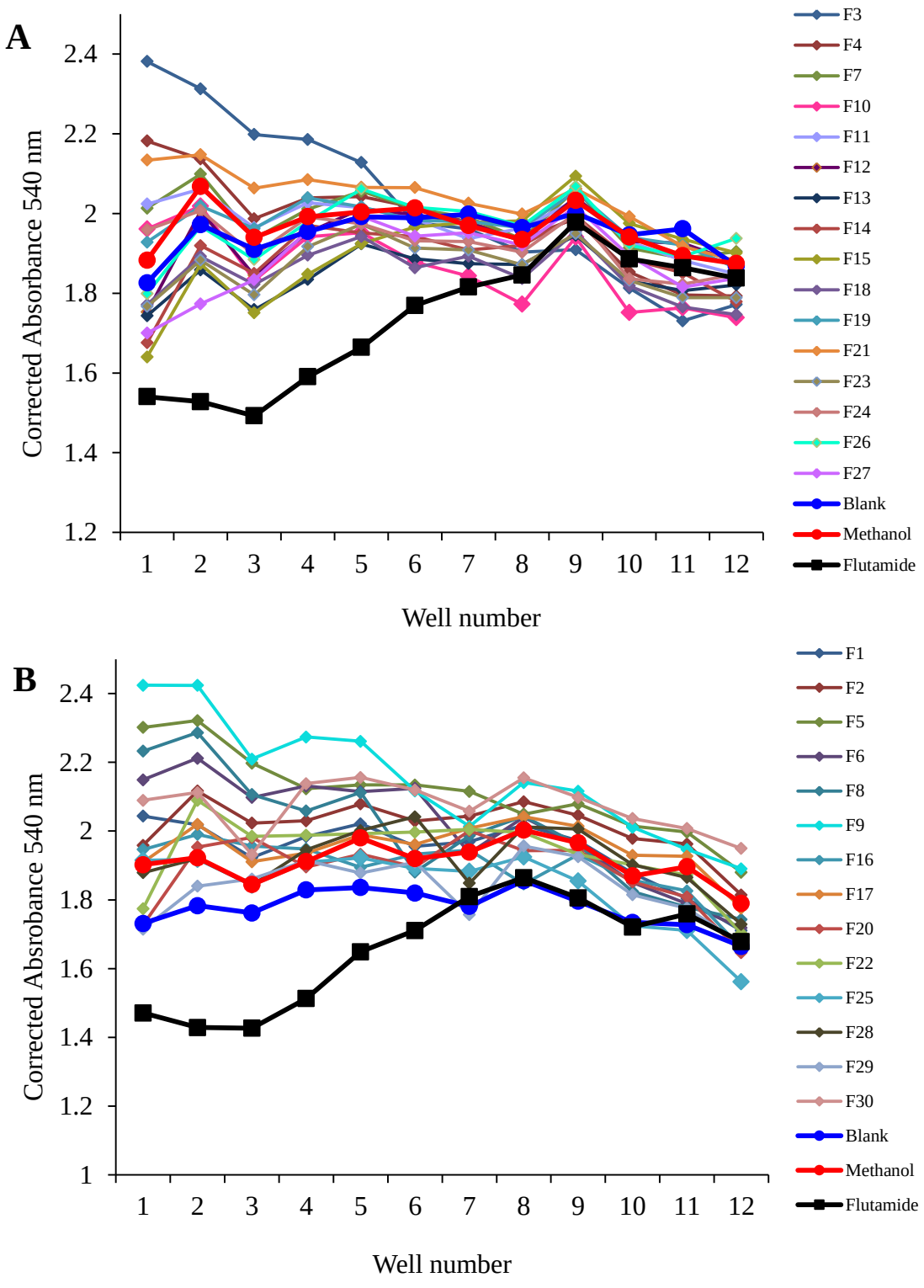

Figure A3.2 *Results from two yeast androgen screens (A&B) assessing fractions from UK3 with a DHT raised background (2 nM) to detect anti-androgenic activity. Flutamide was used as a positive control at concentrations serially diluted from 50 nM (well 1) to 0.02 nM (well 12). A methanol blank was also incorporated in these screens. Fractions were serially diluted from wells 2 to 12, with well 1 containing the pure fraction.*

A3.5 References

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