



Hormones in Treated Sewage Effluent

Final Report

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Executive summary

As part of the Victorian Water Trust's water conservation initiative in 2007, DPI, through the Environmental Health and Chemistry platform's Queenscliff section, was contracted to undertake an assessment of hormones in municipal wastewater treatment plant (WWTP) discharges across the State of Victoria.

The project was conducted between May 2006 and September 2007, and involved the gathering of generic and specific information on all WWTPs in Victoria, and then using that information to produce a list of target WWTPs (i.e. those WWTPs whose effluent we wished to sample) sorted by location (north and south of the state) and treatment type. In all, some 46 WWTPs were chosen for further study of their final discharges. These included lagoon-based plants and those with activated sludge based processes. Permission was obtained from all the relevant water authorities to collect samples of final effluent at point of discharge to the environment, whether that is to a creek, a river, the ocean, or the land.

Samples were collected in August 2006, and then again in late February-early March 2007, and subjected to a number of biological and chemical analyses, including toxicity tests, measurement of hormonal (estrogenic and androgenic) activity using yeast-based bioassays, and the measurement of specific hormonal concentrations using enzyme-linked immunosorbent assays (ELISA).

No sample produced a response in the androgen assay. Lack of assay response was related to lack of androgenic compounds, rather than the direct toxic effect of the effluent. Assessment of testosterone and androstenedione concentrations by ELISA suggests that there were indeed androgens present in the WWTP samples, but at concentrations below 3 ng/L (testosterone), and below 10 ng/L (androstenedione). Although here is very little information on the androgenic activity, or androgen concentrations in WWTP effluents, the concentrations of testosterone and androstenedione observed were comparable with that recently reported in Australia.

Almost all of the effluents examined showed estrogenic activity, to a greater or lesser extent ("not detected" to 62 ng/L estradiol equivalents). On the whole, the levels of estrogenic activity observed were to the lower end of the range observed overseas in the northern hemisphere, and comparable with that recently reported in Australia and New Zealand using similar, human-estrogen receptor based assays ("not detected" to ~ 10 ng/L estradiol equivalents). The reassuring low/no assay response is bolstered by the chemical assessment of estradiol and estrone concentrations by ELISA, which returned concentrations of these compounds for the most part below 10 ng/L.

From an aquatic environmental perspective, it is difficult to say with any certainty what the potential risk to aquatic organisms in waters receiving these effluents will be. Typically, in environmental risk assessment one first looks to agreed national or international guideline or trigger values for the type of waters being assessed. In this case, there are as yet no guideline values. Without guideline values to drive the assessment, then one compares a chemical's concentration in a sample (in this case a WWTP effluent) with data obtained from

toxicological experiments in which the concentration known to elicit a specific effect has been determined. In this case, levels of estradiol were typically between the lowest reported level to induce the production of female-only proteins in male fish (plasma vitellogenin; 1 ng/L), and the lowest concentration of known to induce intersex in fish (8 ng/L). Consequently, such levels in a WWTP discharge are likely to be an environmental risk if there is little or no dilution of the discharge by the receiving water, i.e. discharge represents major component of stream flow. In short, to truly assess the risk (hormonal impact) of these WWTP effluents, *in vivo* testing needs to be undertaken, ideally with a representative native species but failing that with a 'standard' species such as the fathead minnow.

Across Victoria, as indeed elsewhere, a range of different sewage treatment approaches and practices occur at a range of different locations with different climatic influences on WWTP operation. Without marrying explicit details of treatment processes with effluent hormone concentrations, it is difficult to extrapolate results from one location to another. For instance, even with activated sludge treatment, there can be wide differences in key parameters such as hydraulic residence time, and lagoons are potentially exposed to very different temperature regimes affecting performance. The best advice for treatment plant operators is, "have the hormonal activity of your plant measured."

When this program began, the 'watching brief,' being held in Australia on the topic of endocrine disrupting chemicals and their potential effects on aquatic wildlife was considered too passive by many. It still is, by some. Despite the assurance our results may provide (of minimal impact in most cases if there is significant dilution), there is still a need for further extensive on-ground, reassurance research to provide data for higher-level risk assessment by industry and government agencies.

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Glossary of terms

Abiotic: non-living factors of the environment, including light, temperature, inorganic soil particles and rocks, water, and atmospheric gases.

Activated sludge: generic name for an intensive biological treatment of waste in which bacteria are suspended in a tank, and vigorously aerated with a hydraulic retention time (HRT) of 5-20+ hours. The objective of this process is to convert both the soluble and insoluble from a wastewater stream into a flocculant microbial suspension that is readily settleable, e.g. through gravitational solid-liquid separation techniques. Although there are many variations on the process, typically, a portion of the settled biological sludge is returned to the head aeration tank with additional incoming waste.

Agrochemical: for the purpose of this report an agrochemical includes any substance or organism used to:

- Destroy, stupefy, repel, inhibit the feeding of, or prevent pests on plants or other things;
- Destroy a plant or to modify its physiology;
- Modify the effect of another agricultural chemical product; or
- Attract a pest for the purpose of destroying it.

This encompasses all herbicides, insecticides and fungicides. Dairy cleansers for on-farm use, crop markers, insect repellents for use on humans, swimming pool disinfectants, algicides, rodenticides, antifouling paints, timber preservatives, some pest traps and barriers using chemical attractants, and household and home garden products for pest and weed control are also encompassed by the above definition. Fertilisers are not considered an agrochemical for the purposes of this report unless they modify the physiology of a plant.

Androgen: the generic term for any natural or synthetic compound, but usually a steroid hormone, that stimulates or controls the development and maintenance of masculine characteristics in vertebrates by binding to androgen receptors. This includes the activity of the accessory male sex organs and development of male secondary sex characteristics. Androgens are also the precursor of all estrogens, the female sex hormones. The primary and most well-known androgen is testosterone.

Androstenedione: also known as 4-androstenedione, a steroid hormone produced in the adrenal glands and the gonads as an intermediate step in the biochemical pathway that produces the androgen testosterone, and the estrogens estrone and estradiol.

Androsterone: a metabolite of testosterone, or of progesterone, that also exerts minor masculinising effects, but with one-seventh the intensity of testosterone. It is found in approximately equal amounts in the plasma and urine of both males and females.

ANZECC: Australia and New Zealand Environment and Conservation Council.

Aquatic life: the biological life (e.g. algae, fish, frogs etc.) in or on fresh, marine or estuarine waters (surface or groundwaters).

ARMCANZ: Agriculture and Resource Management Council of Australia and New Zealand.

BDL: Below Determination Limit.

Biological filter plants: also known as percolating or trickling (trickle) filter plants, generally comprise a tank with a biofilm supported on coarse media upon which the sewage liquor

(post-primary sedimentation) is sprayed. The water contact time with the biofilm is often quite short, around 30 min. To improve the effluent quality an increasing number of BFP have some form of tertiary treatment.

Contaminant: a chemical that is present in the environment as a consequence of anthropogenic activity. A material described as a 'contaminant' is one that is either not naturally present in the environment being examined, or is present in unnatural concentrations. However, in being described as a contaminant, no judgement is being made about whether or not the material is having an adverse effect on the environment, or organisms therein – the material is simply present in the environment.

Dihydrotestosterone: 5 α -Dihydrotestosterone, or DHT is a biologically active metabolite of the steroid hormone testosterone, formed primarily in the prostate gland, testes, hair follicles, and adrenal glands by the enzyme 5 α -reductase.

Drinking water: water suitable for human consumption without deleterious health risk. Synonymous with 'potable water,' but the preferred term since it is better understood by the community at large.

Endocrine disrupting chemicals: Exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body (of a human and/or wildlife species) responsible for the maintenance of homeostasis and the regulation of developmental processes (Kavlock, 1999). Also defined as:

- Exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or in its progeny, or (sub)-populations (WHO/IPCS, 2002).

Environmental hazard: anything with the potential to cause injury, illness and damage to both living and non-living things within the environment. A danger posed to the environment, whether imminent or otherwise, resulting from any activities, practices, the location, storage or handling of any substance having toxic, corrosive, flammable, explosive, infectious or otherwise dangerous characteristics (adopted from the Environment Protection Act 1970 (Vic), Section 4).

Environmental impact: any impact on plants, animals or the environment caused by human activities is an environmental impact. Impacts may be reversible or irreversible, minor or major, affect a whole ecological community or only a few individuals.

Environmental impact assessment: environmental impact assessment (EIA), also called ecological risk assessment (ERA), is the practice of measuring or estimating the nature and likelihood of effects of an action (e.g. the application of pest control products or practices) on one or more environmental parameters.

Estradiol: also oestradiol, or 17 β -estradiol, this is the major sex hormone in female vertebrates, although it is also produced by males. Estradiol represents the major estrogen in humans. Estradiol has not only a critical impact on reproductive and sexual functioning, but also affects other organs including bone structure.

Estriol: also oestriol, is one of the three main estrogens produced by humans, although this steroid hormone is only produced in significant amounts during pregnancy (as it is made by the placenta).

Estrone: also oestrone, is an estrogenic steroid hormone derived from androstenedione secreted by the ovary. The least prevalent of the three major steroid estrogens (estradiol being most prevalent), estrone is relevant to health and disease due to its conversion to estrone sulfate, a long-lived derivative that acts as a pool of estrone which can be converted as needed to the more active estradiol. Estrone enters a wastewater treatment system either directly from excretion of humans (in the free form or as glucuronide or sulfate conjugates) or from the oxidation of 17 β -estradiol in the treatment plant itself.

Guideline: Numerical concentration limit or narrative statement to support and maintain designated water use.

In vivo: (biological) process occurring or made to occur within a living organism or natural setting.

In vitro: (biological) process made to occur in a laboratory vessel or other controlled experimental environment rather than within a living organism or natural setting.

LOR: Limit of Reporting.

Pesticide: see 'agrochemical.'

Potable water: water suitable for human consumption without deleterious health risks.

Recycled water: water recycled from the effluent of sewage treatment plants (synonymous with reclaimed water).

Reclaimed water: water which, as a result of treatment of waste, is suitable for a direct beneficial use or a controlled use that would not otherwise occur (synonymous with recycled water).

Sediment: Unconsolidated mineral and organic particulate material that has settled to the bottom of aquatic environments.

Teratogenicity: the potential of a chemical to cause structural malformations or defects in offspring; the production of structural malformations or defects in offspring (IUPAC, 1993).

Testosterone: the principal male sex hormone of vertebrates. A steroid hormone, testosterone is derived from cholesterol. The largest amounts of testosterone are produced by the testes in males. It is also synthesized in far smaller quantities in females by the thecal cells of the ovaries, by the placenta, as well as by the zona reticularis of the adrenal cortex in both sexes. In both males and females, testosterone plays key roles in health and well-being, e.g. enhanced libido, energy, immune function, and protection against osteoporosis.

Toxicant: A chemical that can produce adverse health effects.

Trickle filters: see biological filter plants.

Water recycling: the preferred term for generic water reclamation and reuse in Australia, although also defined as the reclamation of effluent generated by a given user for on-site use by the same user (such as in industry).

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Our appreciation is extended to those individuals from government and industry who contributed their time and valuable knowledge at Project Steering Group meetings, and in assisting with sample collection.

1. Introduction

The last several decades has seen concerns raised that a range of chemicals found in municipal wastewater treatment plant (WWTP) discharges can cause adverse environmental impacts. More recently, the occurrence of endocrine disrupting chemicals (EDCs) in WWTP discharges and their impact on aquatic wildlife has generated a significant amount of scientific and public interest since the publication of the book *Our Stolen Future* ¹. Since then, substantial evidence has emerged that many chemicals induce hormone-like effects in wildlife and humans at the concentrations observed in the environment (concentrations much lower than those used in toxicity tests designed to see if the chemicals cause cancer) ².

- The effluent from municipal WWTPs is considered the source of much of the EDC input into aquatic environments.
- Some of the most commonly studied compounds found in WWTP discharges that show hormonal activity are the natural and synthetic estrogens, such as 17 β -estradiol and 17 α -ethynyl-estradiol.

Compared to Europe, Japan and North America, there is still very little information on the overall level of estrogenic activity, or concentrations of specific hormonal compounds WWTP discharges, including recycled water in Australia. The information that is most readily available is variable, ranging from reports of very low concentrations in Queensland, to values in Victoria in line with overseas experience ³.

- This discrepancy between experiences across the country is of both scientific and practical interest to water managers.

As part of the Victorian Water Trust's water conservation initiative in 2007, DPI, through the Environmental Health and

Chemistry platform's Queenscliff section, was contracted to undertake an assessment of hormones in municipal wastewater treatment plant discharges across the State of Victoria.

The specific objectives of the project were:

- To measure the hormonal activity and concentration of several specific natural hormones (the estrogens estradiol and estrone, and the androgens testosterone, and androstenedione) in a range of Victorian WWTP discharges in the Victorian summer and winter.
- To assess the risk to aquatic environments from the hormonal activity found in the project surveys.
- To compare the hormonal activity and specific estrogen / androgen concentrations with those found elsewhere nationally and internationally.

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2. Background

The occurrence of endocrine disrupting chemicals (EDCs) in the aquatic environment and their impact on indigenous fauna has generated a significant amount of scientific and public interest since the publication of the book *Our Stolen Future*¹. Since then, substantial evidence has emerged that many chemicals induce hormone-like effects in wildlife and humans², at the concentrations observed in the environment, i.e. at concentrations much lower than those used in toxicity tests designed to see if the chemicals cause cancer. Chemicals with hormonal activity, i.e. potential endocrine disrupters, include:

- Natural hormones. These can be from any animal, and once released into the environment, chemicals produced by one species can exert hormonal actions on other animals, e.g. human hormones unintentionally reactivated during the processing of human waste in sewage effluent, may result in changes to fish.
- Natural chemicals, including toxins produced by components of plants (phytoestrogens, such as genistein or coumestrol) and certain fungi.
- Synthetically produced pharmaceuticals that are intended to be highly hormonally active, e.g. components of the contraceptive pill and treatments for hormone-responsive cancers.
- Man-made chemicals and by-products released into the environment.

Estrogen mimics are not the only class of endocrine disruptors. Some chemicals antagonise male hormones, i.e. they are androgenic. Indeed, perversely, some compounds may be both estrogen agonists and androgen antagonists, e.g. DDE³.

The endocrine and reproductive effects of EDCs are thought to be due to their ability to (1) mimic, (2) antagonise, or (3) disrupt

the synthesis and metabolism of hormones, or (4) disrupt the synthesis and metabolism of hormone receptors. The discovery of this hormone-like activity occurred long after the release of chemicals into the environment³, yet for most associations between exposure to EDCs and subsequent biological outcomes, the mechanism(s) of action are still poorly understood². This can make it difficult to distinguish between both direct and indirect effects of exposure to EDCs, and primary versus secondary effects.

- Some studies assume that EDCs only produce effects on development rate, growth and reproduction by disrupting hormones controlling reproduction and maturation, but life-history traits may be the result of toxic effects, or sex-related differences in sensitivity (i.e. survival, condition).
- To truly assess the endocrine disrupting effects of chemicals in fish, for instance, one would need to ensure that energy intakes have not been affected, i.e. one would need to discriminate between sublethal effects resulting from changed energy intake, and resulting in changed condition, from those related to endocrine disruption *per se*.

The risk to wildlife of exposure to environmental estrogens has been demonstrated in both field and laboratory^{2,4}. Reports of adverse effects (from subtle changes in physiology to permanently altered sexual differentiation) have come from Europe and North America, although in many cases the causal link between endocrine disruption and effect is unclear. There are, however, a number of remarkable and well-publicised examples:

- Male alligators reared in Florida's Lake Apopka have very small penises, an effect linked to chemical residues in their tissues caused by an accident at a chemical plant on the lake's shores⁵.

- Birds of prey exposed to DDT laid eggs with unnaturally thin eggshells, resulting in breakage during laying or incubation, reduced chick survival and ultimately population decline ⁶.
- Embryonic abnormalities have been observed in fish-eating birds, e.g. in the Laurentian Great Lakes, which can be ascribed to PCB exposure ⁷.
- Exposure of marine gastropods to TBT from marine anti-fouling paints causes a masculinisation of female gastropods, and ultimately population decline ⁸.
- The observation of feminised male fish near sewage outlets in several UK and German rivers ^{9,10}.
- Males excrete an estimated 1.6, 3.9 and 1.5 µg/day of E2, E1 and E3, respectively ¹³.
- The picture is more complex in women. Menstruating females excrete an estimated 3.5, 8.0 and 4.8 µg/day of E2, E1, and E3, respectively, but pregnant females excrete two orders of magnitude more hormone (an estimated 259, 600 and 6000 µg/day of E2, E1, and E3, respectively) ¹³.

The effluent from municipal wastewater treatment plants (WWTPs) is considered the source of much of the EDC input into aquatic environments ^{11,12}. Four main classes of EDC have been identified in sewage effluent:

- Natural steroid estrogens (eg. 17β-estradiol, a female sex hormone found in all vertebrates).
- Synthetic estrogens (e.g. 17α-ethinyl estradiol, a major constituent of common oral contraceptives).
- Phytoestrogens (e.g. β-sitosterol, genistein).
- Alkylphenols (e.g. 4-nonylphenol and bis-phenol-A).

Some of the most commonly studied compounds found in WWTP effluents that show hormonal activity are the natural and synthetic estrogens, such as 17β-estradiol and 17α-ethynylestradiol.

- 17β-estradiol (E2), estrone (E1), and estriol (E3) (Fig. 2.1) are excreted by both women and men, mainly as conjugates of sulphuric and glucuronic acids.

Based on excretion studies, it is not unreasonable to expect that the estrogens would be expected in WWTP influents, and to perhaps be found in WWTP discharges. Indeed, estrogenic steroid hormones have been found in WWTP discharges in Europe, the USA, Canada and Japan, and at concentrations as high as 64 ng/L E2, 82 ng/L E1, and 18 ng/L E3 ¹⁴. However, in Australia there has until recently been very little published information on overall hormonal activity (estrogenic or androgenic), or the concentrations of specific hormones in Australian WWTP discharges, or aquatic environments, as most effluent monitoring is concerned with nutrients, metals and organic compounds of industrial origin.

A suite of steroid hormones is likely present in WWTP effluent because the chemical properties that affect removal in WWTPs (e.g. hydrophobicity) and mass loading from human excretion are similar for most of the estrogens, androgens and progestins.

- Although little is known of the fate of androgens in WWTP processes, nor concentrations in WWTP discharges, Kirk et al. ¹⁵ suggest that most of the androgenic activity in municipal wastewater with a predominantly domestic input is from androgens excreted by humans.

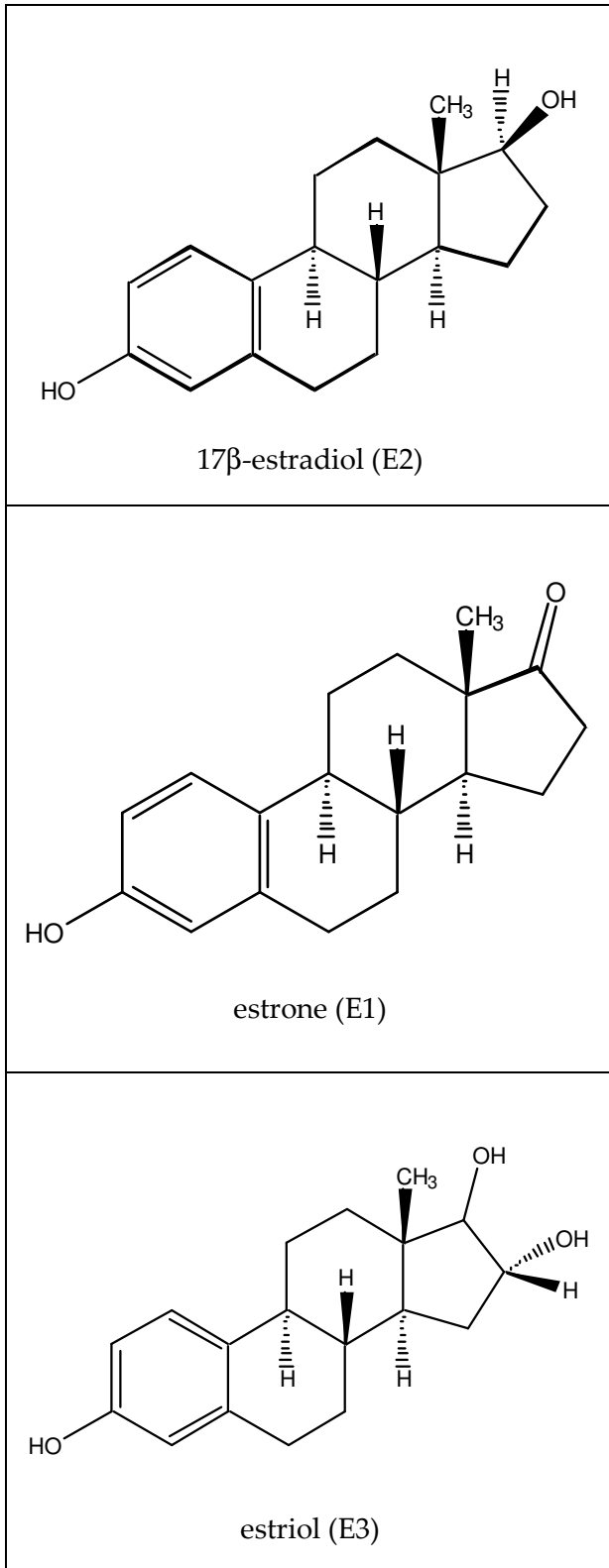


Figure 2.1 Chemical structures of major female sex hormones (estradiol, estrone and estriol).

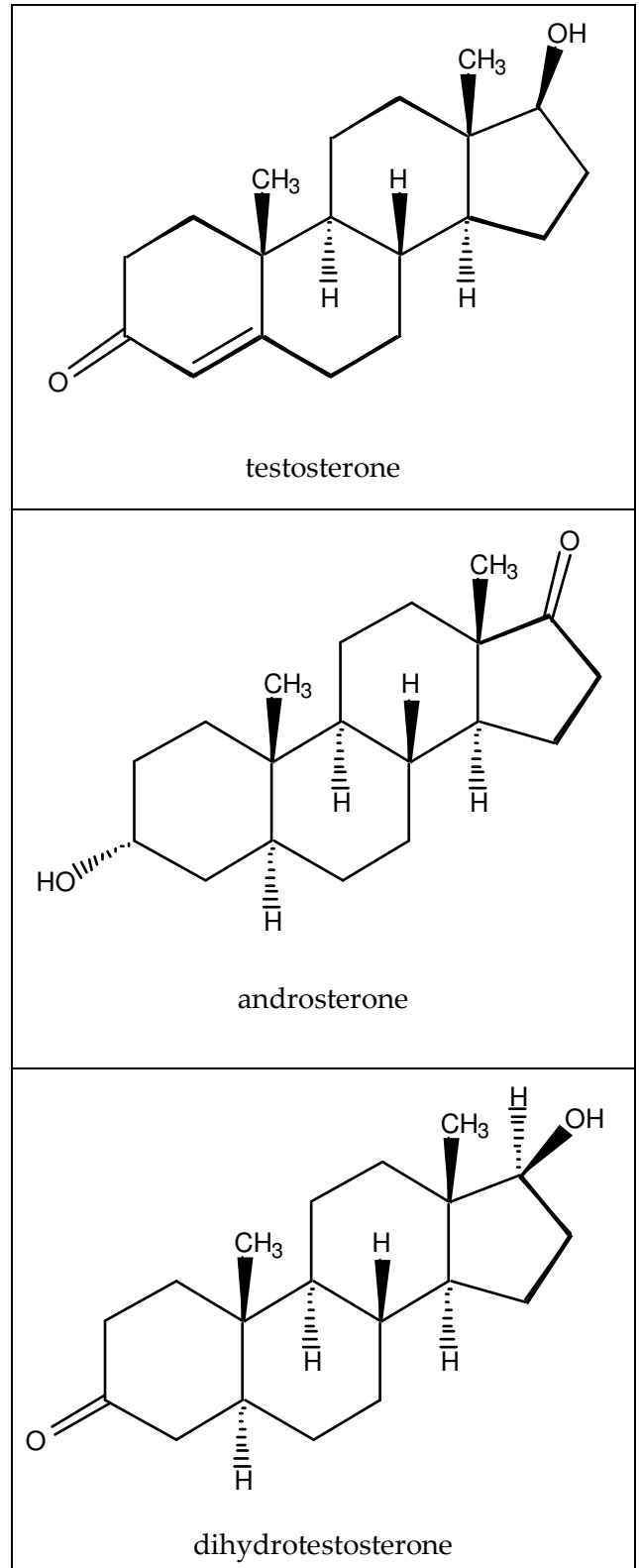


Figure 2.2 Chemical structures of major male sex hormone (testosterone), its major metabolites (androsterone, dihydrotestosterone).

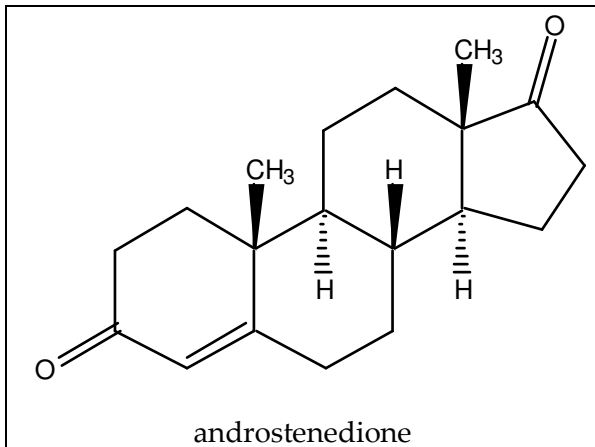


Figure 2.3 Chemical structure of androstenedione, an androgenic steroid hormone and precursor of both testosterone and estradiol.

- Chapman et al. ¹¹ suggest that androgen levels in human plasma are several hundredfold higher than estrogen levels in adult males (3000-10000 ng/L testosterone cf. 10-60 ng/L estradiol). Testosterone concentrations in adult females are of the same order of magnitude in adult females (200-750 ng/L testosterone cf. 30-400 ng/L estradiol (Tietz (1987), as cited in ¹¹).

The shortage of information in Australia on estrogens in Australian WWTP effluents was partly addressed by the publication in 2005 of the results of our reconnaissance survey of the estrogenic activity of seven Victorian WWTP discharges undertaken in 2003¹⁶.

- Estrogenic activity was found in all samples (<0.5 – 55 ng/L estradiol equivalents (EEQ))

In a second survey in early 2004, we again assessed the estrogenic activity of treated municipal wastewater, this time from twelve WWTPs located in southern Victoria and south-eastern South Australia, including all seven original WWTPs ¹⁷.

- The levels of estrogenic activity seen (<0.5 – 45 ng/L estradiol equivalents

(EEQ)) were comparable to those reported in our 2003 survey, i.e. where low concentrations were observed in 2003, low levels were observed in 2004. Similarly where high concentrations were observed in 2003, high levels were observed in 2004.

In 2003 and 2004, the discharge with the highest estrogenic activity was not from the largest population. Indeed, it is one of the smallest. This is consistent with overseas observations. Differences in treatment plant catchment characteristics, including population served, commerce-industry-domestic sewerage mix, treatment technology used and other socio-economic factors may influence the influent, and effluent concentration of endocrine disrupting chemicals ¹². Climatic differences can complicate comparisons, or use of data from one region to another. For instance, temperature is thought to affect the rate of degradation of hormones in activated sludge plants ¹¹. That said:

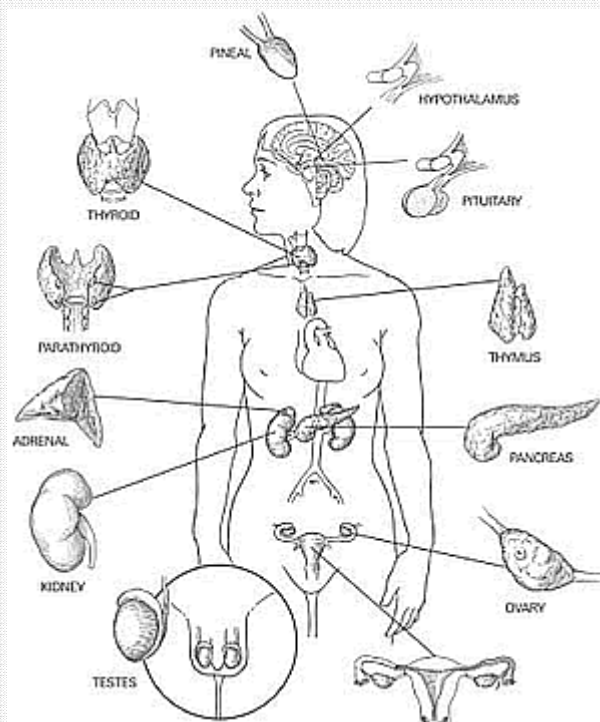
- In both our 2003 and 2004 surveys, the estrogenic activity observed was comparable with, but generally to the lower end of, the range observed overseas e.g. in Japan (5-15 ng/L EEQ) ¹⁸, the USA (21-147 ng/L EEQ) ¹⁹, Sweden (<0.1-15 EEQ) ²⁰, and Switzerland (<1-90 ng/L EEQ) ²¹, respectively.
- In both our 2003 and 2004 surveys, the estrogenic activity observed was higher than that observed in samples obtained in 2003 from 15 municipal sewage treatment plants in Queensland and New Zealand ²². Of these fifteen plants, thirteen effluents were reported as having activity <1 ng/L EEQ, with one suspended growth/activated sludge plant having an activity of 4.2 ng/L EEQ, and one trickle filter plant effluent having an activity of 6.4 ng/L EEQ ^{22,11}.

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Endocrinology 101: The Endocrine System



The human endocrine system (from [Endo 101, 2006](#))

The endocrine system is one of the body's main systems for communicating, controlling and coordinating the body's work. In other words, the endocrine system is a network of glands, hormones and receptors that provides the communication and control links between the nervous system, and bodily functions such as reproduction, immunity, metabolism and behaviour. The endocrine system has three main components:

- **Endocrine glands:** situated at various sites around the body, and in specialised areas of the brain. The cells in these glands secrete specific chemicals called hormones.
- **Hormones:** chemicals that circulate around the body via the blood stream and modulate cellular or organ functions by binding with receptors in the target cells. Hormones that stimulate and

control the activity of other endocrine glands are called trophic hormones.

- **Receptors:** in the target cells that, once activated by binding of the hormone, regulate the functions and processes in the tissue through interactions with the cell's DNA or other complex intracellular signalling processes.

Although the precise structures and roles of the various organs and hormones differ between different vertebrates, particularly in relation to the different life cycle and development stages in different species, the endocrine systems of all vertebrates are similar to that of humans. Hence, the concern that harmful effects seen in wildlife following exposure to endocrine disrupting chemicals (EDCs) may eventually be seen in humans (and *vice versa*).

- Invertebrates, such as molluscs, crustaceans and insects, also have endocrine systems that control a similar range of body functions, although these have evolved along markedly different lines to those of vertebrates.

The endocrine system plays an essential and all-pervasive role in the regulation of metabolic processes. In other words, this is a system that works with the nervous system, reproductive system, kidneys, gut, liver and fat to help maintain and control body energy levels, and the processes of reproduction, growth and development, homeostasis, and responses to surroundings, stress, and injury.

- To function normally, the body needs glands that work correctly, a blood supply that works well to move hormones through the body to their target points, receptor places on the target cells for the hormones to do their work, and a system for controlling how hormones are produced and used.

The fundamental role of all endocrine systems is to enable the coordinated response of one tissue to signals originating in either another organ or, in some cases, cues originating outside the body.

- For most endocrine systems, the primary objective is to maintain homeostasis, avoiding wild swings in hormone levels or responses (e.g. the maintenance of blood glucose levels by insulin).

To a large extent all endocrine systems operate on a 'seesaw' principle, in which target cells send feedback signals to the regulating cells. If the feedback is negative, secretion of the hormone is altered (usually reduced).

- There are usually refinements to this simple scenario that enable all the body's endocrine systems to be integrated such that organism age, reproductive status, nutritional status, and stress levels are able to override other endocrine systems when danger threatens.
- This is vital for maintenance of good health.

The homeostatic balance must be set (or programmed) before the endocrine system will work correctly. For many of the endocrine systems, it appears that the programming is established during foetal or neonatal development, and that an abnormal environment at this stage can result in permanent misprogramming.

- In mammals, programming of the hypothalamus of the female, but not of the male, to respond to gradually rising estrogen levels by triggering a positive response, is established perinatally, and exposure of the female at this time to moderate levels of male sex steroids will prevent this programming and render the female permanently infertile.

Before sex differentiation, the mammalian embryo has the potential to develop into a male or female phenotype. Following

gonadal sex differentiation, perinatal testosterone secretion by the testis is responsible for masculinisation of the body in general. Females avoid developing as a male by not switching on secretion of testosterone in the ovary. The central role of testosterone in masculinisation has two important implications:

1. If a genotypic male fails to secrete sufficient testosterone, it will not masculinise and may develop as a phenotypic female (but with testes).
2. If a genotypic female is exposed to sufficient testosterone (or other androgen), it will masculinise and may develop as a phenotypic male (but with ovaries).

These are not always all or nothing scenarios. Partial masculinisation, or partial failure of masculinisation, can also occur.

Perhaps the most important aspects of these, and other imprinting/programming changes is their irreversibility, leading to perhaps the greatest concern about environmental endocrine disruptors, namely that exposure to an agent during perinatal life can result in permanent adverse or abnormal change. The exposure does not need to be chronic, simply sufficient at a critical time during development.

Non-mammalian vertebrates differ from mammals, and each other, in terms of their reproductive strategies. These include:

- Sequential/simultaneous hermaphroditism.
- Parthenogenesis.
- Viviparity and ovoviviparity.
- Gonochromism.

Breeding frequency may also be more limited, and includes the semelparous (breed only once) as well as iteroparous

(breed two or more times). However, the endocrine axes non-mammalian vertebrates are similar to that of mammals in their operation, in the pattern of feedback mechanisms and in the hormones involved.

For instance:

- In many male teleosts (bony fishes) 11-ketotestosterone is the predominant circulating androgen (cf. testosterone in mammals).
- Female teleosts produce testosterone, and at times circulating levels may be as high as that of estradiol.
- Teleosts also produce a range of progesterone-like molecules that cause final oocyte maturation and ovulation (although in some teleosts the corticosteroid deoxycorticosterone has the same function).
- Female amphibians also have high levels of circulating androgens as well as estrogens during the reproductive portions of their lives.
- Teleosts have a third estrogen receptor (ER- γ cf. the two found in mammals (ER- α and ER- β)).

Complete or partial sex reversal has been observed when the eggs, larvae or juveniles of non-mammalian vertebrates are exposed to androgens or estrogens. Androgens usually inhibit female duct (Mullerian) development while enhancing male duct (Wolffian) development, while estrogens typically do the reverse. Estrogens stimulate the synthesis of ovalbumin protein in birds, and the synthesis of vitellogenins in adult female vertebrates that produce yolky eggs, i.e. reptiles, birds, amphibians and fish. If adult males or immature females are exposed to estrogens, they can be induced to produce vitellogenins.

- Plasma vitellogenin can be used as a biomarker for exposure to estrogens.

Endocrine axes do not function in isolation from each other, but interact, providing

organisms with the ability to react and adapt to changing circumstance. The cross-talk between endocrine systems is very complex, and new pathways of communication and overlap between the various endocrine systems are being discovered every year. For instance:

- The interaction between leptin and the reproductive system. In general, animals only reproduce only when females have sufficient energy reserves and food supply is good. When food supply and maternal energy (fat) reserves are low, elevated leptin suppresses reproduction.
- Pathways such as this play important roles in the timing of puberty, in regulating seasonal reproduction, and even in disorders such as anorexia.

Cross-talk between the endocrine systems can have different consequences at different stages of life, and in particular during the phase when an endocrine axis is being programmed, i.e. when thresholds for stimulation and feedback loops are being created.

Adapted from:

1. WHO/IPCS (2002). Global assessment of the state-of-the-science of endocrine disruptors. Edited by: Damstra T, Barlow S, Bergman A, Kavlock R, Van Der Kraak G. World Health Organisation / International Program on Chemical Safety. Available on-line: www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/index.html
2. Endo 101 (2006). Endo 101: the endocrine system. The Hormone Foundation. Available on-line: www.hormone.org/endo101/
3. Stryer L (1995). Biochemistry. 4th Ed. W.H. Freeman & Co., New York.

3. Material and Methods

The project was overseen and guided by a Project Steering Committee comprised of representatives of:

- Department of Sustainability and Environment (for the Victorian Water trust)
- Department of Primary Industries
- Melbourne Water
- Wannon Water
- South-East Water
- Environment Protection Authority Victoria
- Corangamite Catchment Management Authority

The project was managed by DPI, and delivered by Environmental Health & Chemistry's Queenscliff Section, in collaboration with Dr. Fujio Shiraishi of the National Institute for Environmental Studies, Japan, and Dr. Scott Salzman of Deakin University.

In its early stages, the project was faced with a choice, whether to conduct in-depth investigations on a limited number of WWTPs (i.e. from raw influent to treated effluent), or to take a broader look at a number of WWTPS (i.e. measure hormonal activity at a large number of locations).

- After much discussion, the Project Steering Committee decided to take the latter approach, in part justifying the decision on the paucity of information on hormonal activity of Victorian WWTP discharges.

The Project Steering Committee also suggested that the project should investigate treatment type and temperature because they are considered two of the major variables influencing persistence/degradation of hormones in the treatment process.

- Although most of Victoria's population, and hence most of the biggest WWTPs, are south of the Dividing Range,

available information suggested there were sufficient numbers of WWTPs north of the Dividing Range to allow plants to be grouped by general location (as surrogate for temperature), as well as treatment types.

Basic information on WWTPs (e.g. treatment type), was obtained from published information on water business websites and in reports, and/or directly from the water authorities. Using this information, we ascertained that, although there are some 185 WWTPs in the state, there were:

- (a) An insufficient number of activated sludge plants with BNR in the state to allow us to sub-divide the activated sludge category as we had hoped;
- (b) There were only nine WWTPs using activated sludge systems of any description in our 'north';
- (c) There were plants using activated sludge processes with extended aeration (IDEA process) that formed a category of their own.

Consequently, a primary list of targets was compiled, that included:

- Activated sludge-based plants (AS; S (south)=13, N(north)=5)
- Activated sludge (extended aeration)-based plants (AS(EA); S=7, N=4).
- Lagoon based plants (L; S=8, N=8).
- Other – measured because of their importance to a particular water authority, but not included in the statistical comparisons because of the use of a different process to the other plants in our survey (1).

Chemical and biochemical analyses included:

- Toxicity.
- Hormonal activity (e.g. estrogenic, androgenic activity).

- Specific hormone concentrations (estradiol, estrone, testosterone, androstenedione).
- pH, electrical conductivity, TOC (total organic carbon), chlorophyll 'a' (Summer 2007, only).

3.1 Sample Collection

Two surveys were conducted of an intended 46 WWTPs:

- Phase 1 (Winter 2006) in the three weeks between August 14 and 31, 2006.
- Phase 2 (Summer 2007) in the four weeks from February 12 – March 7, 2007.
- Samples could not be obtained from one WWTP in the Winter 2006 survey for logistical reasons, and was removed from the Summer 2007 survey.
- Samples could not be obtained from five WWTPs in the Summer 2007 survey, also for logistical reasons. The Winter 2006 data has been retained in statistical comparisons.

Water samples were collected as 'grab,' or spot samples, from each facility at the point at which effluent enters the environment, either as recycled water or direct discharge to the receiving water.

- In Winter 2006, 1.5-2.5L of water was collected.
- In Summer 2007, 5-6L of water was collected (to facilitate additional method development and testing not reported herein).

Although for the most part discharges were sampled in the same manner, at times sampling protocols were modified dependent upon on-site conditions. For instance, generally samples were collected by members of the research team, although some samples were collected by plant personnel (for safety reasons or convenience). Samples were directly collected in glass bottles, stored on ice, and then at 4°C until processed.

3.2 Sample preparation: bioassay

For each WWTP site, a sample (1L) was extracted for the measurement of hormonal activity (estrogenic, androgenic and retinoic) activity and toxicity, using the yeast two-hybrid assay and photobacterium toxicity assay.

Prior to filtration or extraction, 10mL of an acetic acid: water: methanol (1:9:90) buffer solution was added to the sample (1 L) to ensure an acid pH. Samples were then filtered with GF/C filters (Whatman International Ltd, UK) to remove particulate matter. The sample was then passed through a C18 SPE disk (octadecyl C18; Empore; 47 mm; 3M, MN, USA), which had previously been conditioned with sequential washes of methanol (10 mL), and deionised water (water having a resistivity of at least 18 MΩ cm⁻¹ produced by passing singly distilled water through a Milli-Q Water Purification System; 20 mL). Thereafter, the disks were immediately washed with deionised water (20 mL), and dried at 35°C on a hotplate for approximately 1.5 hours. Each disk was wrapped separately in aluminium foil, labelled, placed inside another labelled plastic bag, and stored at -4°C until transported to Japan for analyte elution and analysis.

Each disk was eluted with methanol (10 mL) into a screw cap glass tube (Iwaki borosilicate glass; Asahi Techno Glass, Tokyo), and the resulting solution evaporated to dryness with nitrogen, and the residue immediately frozen (-20° C) until separation the following day. The sample was then subjected to a crude fractionation process to isolate fractions containing non-polar chemicals (e.g. many of the persistent organic pollutants), the steroid hormones, and polar compounds.

The sample was re-suspended in a mixture of 3:1 hexane: dichloromethane (1

mL), and loaded onto a florisil column (Varian Bond Elut-FL, 500 mg, 3mL; CA, USA) which had previously been conditioned with hexane (2.5 mL). After loading of the sample onto the column, the column was washed twice with a mixture of 3:1 hexane: dichloromethane (2.5 mL). The sample solution passing through the florisil column and the collected washes were combined and designated the W fraction. Thereafter, the cartridge was washed with a mixture of 1:9 acetone: dichloromethane (2 x 2.5 mL). The collected washes were combined and designated the FL fraction. Finally, the cartridge was washed with methanol (2 x 2.5 mL), and the collected wash designated the RM fraction. The W, FL and RM fractions were evaporated to dryness under nitrogen, and frozen (-20°C) until subjected to a number of analyses:

- Modified photobacterium toxicity test.
- Yeast human androgen receptor (hAR) bioassay.
- Yeast human estrogen receptor (hER) bioassay.
- Yeast medaka estrogen receptor (mER) bioassay.
- Yeast retinoic acid receptor (RAR) bioassay (*data not reported*).
- Yeast aryl hydrocarbon receptor (AhR) bioassay (*data not reported*).

3.3 Measurement of hormonal activity with yeast two-hybrid assay incorporating estrogen / androgen receptors

Samples and standards were removed from the freezer 1h prior to analysis, thawed, and dissolved in DMSO (100µL), effectively resulting in 10,000-fold concentration from the original effluent sample.

The agonist activities of the treated municipal effluent samples were measured with a yeast two-hybrid estrogenicity assay system using yeast cells (*Saccharomyces*

cerevisiae Y190) into which the human estrogen receptor ER α or the estrogen receptor from Japanese medaka (*Oryzias latipes*) had been inserted. Both were adapted to a chemiluminescent reported gene (for β -galactosidase) method employing a 96-well culture plate ¹.

In short, yeast cells were cultured (30°C, overnight; Sanyo Incubator, Tokyo, Japan) in a modified SD (Sabouraud Dextrose) medium (lacking tryptophan and leucine). The yeast solution cell density was measured (595 nm), and, if necessary, adjusted to 1.75 -1.85 readings for constant cell density by diluting with modified SD medium. Modified SD solution (60 µL) was added to each well of the first row of a 96-well culture plate (Sumilon 96F disposable plates; Sumilon Bakelite Co., Tokyo, Japan). Thereafter, 2% DMSO / modified SD solution (60 µL) was automatically added (Nichiryo NSP-7000 Multi-channel Auto Sampling System, Nichiryo Co., Tokyo, Japan) to each well of the 2nd - 8th rows of the plate. Six samples were run on each plate, with aliquots of each sample (60 µL), added to two, neighbouring wells of the 1st row of the plate. An aliquot was removed from each well of row 1, and added to row 2 to dilute 2-fold. This process was then repeated from rows 2-7. No sample solution from row 7 was added to the 8th row. Thereafter, yeast solution (60 µL) was added to all wells, the plate shaken (30s; Taiyo S-2000 Automatic Mixer, Taiyo, Tokyo, Japan) and then incubated (30°C, 4 h).

After incubation, a mixed solution (80 µL) for inducing chemiluminescence and for enzymatic digestion (30 µL of Aurora GAL-XE Reaction Buffer containing GalactaLux substrate, MP Biomedicals Inc., CA, USA and 50 µL of Zymolyase 20T diluted with Z buffer (a mixture of 21.5 g Na₂HPO₄.12H₂O; 6.2 g Na₂HPO₄.

.2H₂O; 0.75g KCl; 0.246 g MgSO₄·7H₂O in 1L deionised water)) was then added to each well, and the plate incubated (37°C, 1 h; Ikemoto Scientific Technology Co, Tokyo, Japan). Thereafter, a light emission accelerator solution (50 µL; Aurora Accelerator, MP Biomedicals Inc., CA, USA) was added to each well, and the chemiluminescence produced by released β-galactosidase measured with a 96-well plate luminometer (Luminescencer-JNR AB-2100, ATTO Bioinstruments, Tokyo, Japan). Agonist activity was recorded as the EC × 10 (defined as the concentration of test solution producing a chemiluminescent signal 10 times that of the blank (negative) control). Two positive controls were used, namely 17β-estradiol and estrone (Wako Pure Chemical Industries Ltd, Osaka, Japan) in both the mER and hER assays, and dihydrotestosterone and 11-ketotestosterone (Wako Pure Chemical Industries Ltd, Osaka, Japan) in the hAR assay. A solvent (vehicle) control (DMSO, Nakalai Tesque Co., Kyoto, Japan) was also used.

3.4 Measurement of toxicity using a modified photobacterium toxicity test

The Microtox acute toxicity, screening test (developed by Beckman Instruments, Inc.) was adapted for use with a 96-well microplate, and from here-on is referred to as the photobacterium toxicity test (P.B.). This bioassay is based on the production of light per unit time by living luminescent bacteria, which is a reflection of the rate at which a complex set of energy-producing reactions is operating. Chemical inhibition of any of the enzymes will alter this rate, consequently changing the amount of light produced. Chemical inhibition is determined by measuring the IC₅₀ (the chemical concentration calculated to inhibit luminescence in 50% of the test organisms). In this study, the IC₅₀ values are reported

according to a concentration ratio (C.R.), which is effectively how much the sample would have had to have been diluted to inhibit luminescence in 50% of the photobacteria. In short, the lower the IC₅₀ (C.R.) reported, the higher the toxicity of the sample (and *vice versa*, i.e. the higher the IC₅₀ (C.R.), the lower the toxicity).

Photobacterium cells were cultured at room temperature overnight in an equal mixture of Marine Broth medium and T medium (peptone, 0.4 g; glycerol 3.5 g; NaCl 20 g; MgSO₄·7H₂O, 29 g; KCl, 0.9 g; K₂HPO₄, 0.1 g; 1M MOPS buffer solution, 4.5 mL). The bacteria solution was well mixed, and its luminescence intensity measured for adjustment by placing an aliquot of the mixture (200 µL) into a black 96 microplate and reading the intensity (Luminescencer-JNR AB-2100, ATTO Bioinstruments, Tokyo, Japan). The bacterial mixture is deemed acceptable if the measured intensity is greater than 200,000.

After adding equal amounts of 10% glycerol T medium to the bacterial solution, the resulting mixture was diluted 10 times by adding T medium (60 µL) to each well of the first row of a 96-well culture plate (Sumilon 96F disposable plates; Sumilon Bakelite Co., Tokyo, Japan). Thereafter, 2% DMSO / T med. solution (60 µL) was automatically added (Nichiryo NSP-7000 Multi-channel Auto Sampling System, Nichiryo Co., Tokyo, Japan) to each well of the 2nd - 8th rows of the plate.

Test samples (from extraction process described earlier (3.2)) are resuspended in DMSO (100 µL). An aliquot of the resuspension (20 µL) is then mixed with T medium (480 µL), effectively diluting the sample 25-fold, and providing a 4% DMSO solution for testing.

- Diluting the sample at this point effectively reduces sample pre-concentration (i.e. from grab sample to testing) to 400-fold.

Five samples plus a control (4% DMSO), were run on each plate. In short, aliquots of each sample (or control; 60 μ L) were added to two, neighbouring wells of the 1st row of the plate. An aliquot was removed from each well of row 1, and added to row 2 to dilute 2-fold. This process was then repeated from rows 2–7. No sample solution from row 7 was added to the 8th row. Thereafter, photobacterium solution (60 μ L) was added to all wells, the plate shaken (30s; Taiyo S-2000 Automatic Mixer, Taiyo, Tokyo, Japan) and after five minutes the chemiluminescence measured with a 96-well plate luminometer (Luminescencer-JNR AB-2100, ATTO Bioinstruments, Tokyo, Japan).

3.5 Measurement of hormone concentrations using ELISA

3.5.1 Sample preparation and extraction

For each WWTP site, a sample (500 mL) was extracted for the measurement of the concentration of four, specific hormones (estradiol, estrone, testosterone, androstenedione) using commercial enzyme-linked immunosorbent assay (ELISA) kits.

Prior to filtration, 5mL of an acetic acid: water: methanol (1:9:90) buffer solution was added to the sample to ensure an acid pH. Samples were then filtered with GF/C filters (Whatman International Ltd, UK) to remove particulate matter. The sample was then passed through a C18 SPE column (Discovery DSC-18, 6 mL 500mg, Supelco, PA USA), which had previously been conditioned with sequential washes of methanol (6 mL), and deionised water (6 mL). Thereafter, the columns were

immediately washed with deionised water (water having a resistivity of at least 18 M Ω cm⁻¹ produced by passing singly distilled water through a Milli-Q Water Purification System; 50 mL), and eluted with methanol (5 mL) into a screw cap glass tube (Iwaki borosilicate glass; Asahi Techno Glass, Tokyo), and the resulting solution evaporated to dryness with nitrogen.

Each sample was subjected to a crude fractionation process to isolate fractions containing non-polar chemicals (e.g. many of the persistent organic pollutants), the steroid hormones, and polar compounds.

In short, the sample was re-suspended in a mixture of 3:1 hexane: dichloromethane (1 mL), and then loaded onto a florisil column (Strata FL-PR Florisil, 500 mg, 3 mL; Phenomenex, USA) which had previously been conditioned with hexane (2.5 mL). After loading of the sample onto the column, the column was washed twice with a mixture of 3:1 hexane: dichloromethane (2.5 mL). The sample solution passing through the florisil cartridge at this time (the W fraction) was discarded. Thereafter, the cartridge was washed with a mixture of 1:9 acetone: dichloromethane (2 \times 2.5 mL). The collected washes were combined and designated the FL fraction. The FL fraction was evaporated to dryness under nitrogen, resuspended with 10 μ L DMSO, 100 μ L methanol and 890 μ L of water, and frozen (-20°C) until subjected to a number of ELISA measurements, specifically for:

- Estradiol
- Estrone
- Testosterone
- Androstenedione
- Total estrogenic activity (*data not reported*)

3.5.2 Measurement of estradiol

Measurement of estradiol was conducted using a commercial ELISA kit, namely:

- 17 β -estradiol ELISA. Enzyme-linked immunosorbent assay for the in-vitro-diagnostic quantitative determination of 17 β -estradiol in human serum and plasma (IBL, Hamburg, Germany) (IBL).

Note: The IBL kit is principally sold for *in vitro* diagnostic use. However, the principle behind the kits is the same as much more expensive ELISA kits designed for environmental analysis, i.e. competitive binding. In short, a series of microtiter wells are coated with an antibody directed towards a unique antigenic site on the estradiol molecule. Estradiol within a sample competes with a known amount of conjugated estradiol standard for binding to the coated antibody. After a period of incubation, unbound conjugate is washed off the microtiter plate. The amount of bound estradiol conjugate is inversely proportional to the amount of estradiol in the sample. This is quantified by adding a colorimetric substrate to the microtiter plate. After the addition of the substrate, the intensity of colour developed is inversely proportional to the concentration in the sample.

Following sample filtration and extraction (see 3.5) measurement of hormone was conducted in accordance with the manufacturer's instructions, using standards and reagents supplied in the kit. In short, standards, controls, and samples (25 μ L of each; all duplicated) were dispensed individually into wells in the 96-well microplate supplied with the kit. An enzyme conjugate (200 μ L) was then added to each well. After mixing for 10 s, the plate was incubated uncovered for two hours at room temperature. Thereafter, each well of the plate was washed three times with diluted wash solution (400 μ L) using a

microplate washer (ATLANTIS Microplate Washer, ASYS Hitech GmbH, Salzburg, Austria). Substrate solution (100 μ L) was then added to each well, and the plate incubated for 15 minutes at room temperature. The enzymatic reaction was then stopped by adding stop solution (50 μ L), and the absorbance at 450nm in each well read by a microplate reader (UVM 340 Microplate Reader, ASYS Hitech GmbH, Salzburg, Austria) within 10 minutes of adding the stop solution.

3.5.3 Measurement of estrone

Measurement of estrone was conducted using a commercial ELISA kit, namely:

- Estrone ELISA. Enzyme-linked immunosorbent assay for the direct quantitative determination of Estrone in human serum (IBL, Hamburg, Germany) (IBL).

Following sample filtration and extraction (see 3.5) measurement of hormone was conducted in accordance with the manufacturer's instructions, using standards and reagents supplied in the kit. In short, a working solution of the estrone conjugate was prepared by mixing equal volumes of estrone-biotin and avidin-HRP conjugates into the assay buffer (1:100 dilution). This mixture was allowed to stand for 20-25 minutes. Thereafter, aliquots (50 μ L; in duplicate) of each standard, control, and sample was added individually into wells in the 96-well microplate supplied with the kit. Conjugate working solution (100 μ L) was added to each well, and the plate incubated for one hour at room temperature on a plate shaker (UVM 340 Microplate Reader, ASYS Hitech GmbH, Salzburg, Austria). Each well of the plate was then washed three times with diluted wash solution (300 μ L) using a microplate washer (ATLANTIS Microplate Washer, ASYS Hitech GmbH, Salzburg, Austria).

TMB substrate solution (150 μL) was added to each well, and the plate incubated for 10-15 minutes on the microplate shaker at room temperature. The enzymatic reaction was stopped by adding stop solution (50 μL), and the absorbance at 450nm in each well read by a microplate reader (UVM 340 Microplate Reader, ASYS Hitech GmbH, Salzburg, Austria) within 20 minutes of adding the stop solution.

3.5.4 Measurement of testosterone

Measurement of testosterone was conducted using a commercial ELISA kit, namely:

- Testosterone ELISA. Enzyme-linked immunosorbent assay for the in-vitro-diagnostic quantitative determination of testosterone in human serum and plasma (IBL, Hamburg, Germany) (IBL).

Following sample filtration and extraction (see 3.5) measurement of hormone was conducted in accordance with the manufacturer's instructions, using standards and reagents supplied in the kit. In short, standards, controls, and samples (25 μL of each; all duplicated) were dispensed individually into wells in the 96-well microplate supplied with the kit. An enzyme conjugate (200 μL) was then added to each well. After mixing for 10 s, the plate was incubated uncovered for one hour at room temperature. Thereafter, each well of the plate was washed three times with diluted wash solution (400 μL) using a microplate washer (ATLANTIS Microplate Washer, ASYS Hitech GmbH, Salzburg, Austria). Substrate solution (200 μL) was then added to each well, and the plate incubated for 15 minutes at room temperature. The enzymatic reaction was then stopped by adding stop solution (100 μL), and the absorbance at 450nm in each well read by a microplate reader (UVM 340 Microplate Reader, ASYS Hitech GmbH,

Salzburg, Austria) within 10 minutes of adding the stop solution.

3.5.5 Measurement of androstenedione

Measurement of androstenedione was conducted using a commercial ELISA kit, namely:

- Androstenedione ELISA. Enzyme-linked immunosorbent assay for the in-vitro-diagnostic quantitative determination of androstenedione in human serum and plasma (IBL, Hamburg, Germany) (IBL).

Following sample filtration and extraction (see 3.5) measurement of hormone was conducted in accordance with the manufacturer's instructions, using standards and reagents supplied in the kit. In short, standards, controls, and samples (20 μL of each; all duplicated) were dispensed individually into wells in the 96-well microplate supplied with the kit. An enzyme conjugate (200 μL) was then added to each well. After mixing for 10 s, the plate was incubated uncovered for one hour at room temperature. Thereafter, each well of the plate was washed three times with diluted wash solution (400 μL) using a microplate washer (ATLANTIS Microplate Washer, ASYS Hitech GmbH, Salzburg, Austria). Substrate solution (200 μL) was then added to each well, and the plate incubated for 15 minutes at room temperature. The enzymatic reaction was then stopped by adding stop solution (100 μL), and the absorbance at 450nm in each well read by a microplate reader (UVM 340 Microplate Reader, ASYS Hitech GmbH, Salzburg, Austria) within 10 minutes of adding the stop solution.

3.5.6 Calculation of ELISA results

The average absorbance values for each set of standards, controls and samples

were calculated, and a calibration curve constructed by plotting the mean absorbance obtained from each standard against its concentration, using an automated method utilising 4 parameter logistics. The hormone concentration of samples was determined automatically from the calibration curves. Where samples had concentrations higher than that of the highest standard, the samples were diluted further and the concentration determined again. Sample dilution factors were taken into account when calculating hormone concentrations in the WWTP effluent.

3.5.7 QA/QC for ELISA

In order to verify the accuracy and precision of ELISA measurements a number of quality control and assurance procedures were undertaken.

To verify calibration accuracy, check standards (i.e. standards from the kit run as samples) were run in duplicate on each ELISA plate during each ELISA test.

- The ratio of nominal concentrations and result values were 87% (n=10, 71-113%) for 17 β -estradiol, 86% (n=14, 62-139%) for estrone, 83% (n=6, 47-104%) for androstenedione, 87% (n=7, 72-109%) for testosterone, and 83% (n=6, 38-106%) for total estrogen (*data not reported*).

To verify that the hormones in samples were passing through the sample preparation process quantitatively, and to verify that the hormones were found in the expected fractions, a number of recovery experiments were undertaken.

In the first recovery trials, aliquots of kit standards were spiked into deionised water (500 mL) at three concentrations (17 β -estradiol, 0.5, 1.0, 5.0 ng/L; estrone, 0.4, 1.0, 2.0 ng/L; testosterone, 0.3, 1.6, 3.2 ng/L; and androstenedione, 1.0, 5.0, 10.0 ng/L, respectively) on three separate occasions,

and the samples thereafter processed as per WWTP effluents.

- The ratio of nominal concentrations and result values (i.e. recovery) were:
 - 17 β -estradiol, 150% (CV, 12%)
 - 0.5 ng/L, 146%
 - 1.0 ng/L, 161%
 - 5.0 ng/L, 143%
 - Estrone, 118% (CV, 15%)
 - 0.4 ng/L, 112%
 - 1.0 ng/L, 115%
 - 2.0 ng/L, 126%
 - Testosterone, 197% (CV, 21%)
 - 0.3 ng/L, 243%
 - 1.6 ng/L, 164%
 - 3.2 ng/L, 184%
 - Androstenedione, 280% (CV, 18%)
 - 1.0 ng/L, 322%
 - 5.0 ng/L, 292%
 - 10.0 ng/L, 224%

The second recovery trial examined the behaviour of our target hormones in a composite WWTP effluent (prepared by blending equal volumes of five WWTP effluents of the lowest hormonal activity and measured hormone concentrations).

- The hormone concentrations in the composite sample (i.e. background levels) were both estimated from previous measurements and directly measured (n=2);
 - 17 β -estradiol: estimated, 0.1 ng/L; measured, 0.3 ng/L
 - Estrone: estimated, 0.3 ng/L; measured, 0.3 ng/L
 - Testosterone: estimated, 0.2 ng/L; measured, 1.1 ng/L
 - Androstenedione: estimated, 0.0 ng/L; measured, 1.4 ng/L

Aliquots of kit standards were spiked into the composite WWTP effluent (500 mL), and the samples thereafter processed as per WWTP effluents.

- Nominal concentrations (corrected for background hormone concentrations)

were: 17 β -estradiol, 1.7 ng/L; estrone, 1.3 ng/L; testosterone, 2.7 ng/L; and androstenedione, 2.9 ng/L, respectively.

- Measured concentrations: 17 β -estradiol, 2.0 ng/L; estrone, 3.4 ng/L; testosterone, 4.4 ng/L; and androstenedione, 6.9 ng/L, respectively.
- Recoveries: 17 β -estradiol, 118%; estrone, 262%; testosterone, 162%; and androstenedione, 238%, respectively.

In discussing hormone concentrations, data has not been corrected for recovery.

3.6 Statistical analysis

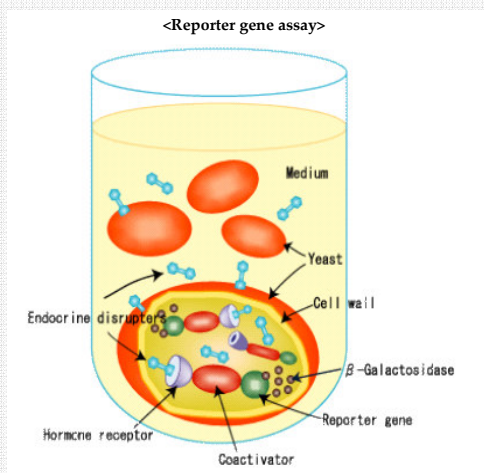
For statistical comparison, where the data did not conform to the stringent assumptions of parametric statistical methodologies, non-parametric statistical methodologies were used instead. For instance, the non-parametric analogue to the one way ANOVA (the Kruskal-Wallis analysis of variance), was used to assess for statistically significant differences in distributions within independent measures with more than two groups. If differences were detected, the Mann-Whitney test (the non-parametric equivalent of the t-test), was used for any subsequent post-hoc comparisons, or where a difference in the distributions in only two groups was investigated. Statistical analysis was conducted using SPSS version 14.0 for windows (SPSS Inc, Chicago) and XLStatistics Ver 5.0.

3.7 References

1. Shiraishi F, Shiraishi H, Nishikawa J, Nishihara T, Morita M (2000). Development of a Simple Operational Estrogenicity Assay System using the Yeast Two-Hybrid System. *Journal of Environmental Chemistry* 10:57-64.

***In Vitro* Assays**

The molecular mechanism of estrogen action is the basis of all *in vitro* tests. The effects of endogenous and/or xenoestrogens are mediated by the estrogen receptor (ER). Inactive ERs exist in large complexes associated with heat shock proteins. When a compound binds to the ER, the heat shock proteins dissociate, and a conformational change activates the receptor and causes dimerisation. The resulting homodimer complex (HDC) shows high affinity for EREs (estrogen response elements) in the regulatory region of estrogen-inducible genes in the nucleus. After binding to the ERE, the HDC recruits transcription factors to the target gene promoter, leading to gene activation and transcription, and subsequent translation of RNA into the proteins that ultimately stimulate the observed responses.



Several *in vitro* assays have been developed to screen the estrogenic activity of compounds in freshwaters or waste-water treatment plant effluents.

- **ER (Estrogen Receptor) competitive ligand binding assays** quantify the ability of a compound to compete with estradiol (E2) to bind to the estrogen receptor (ER).
- **Cell proliferation assays** measure the increase in numbers of estrogen

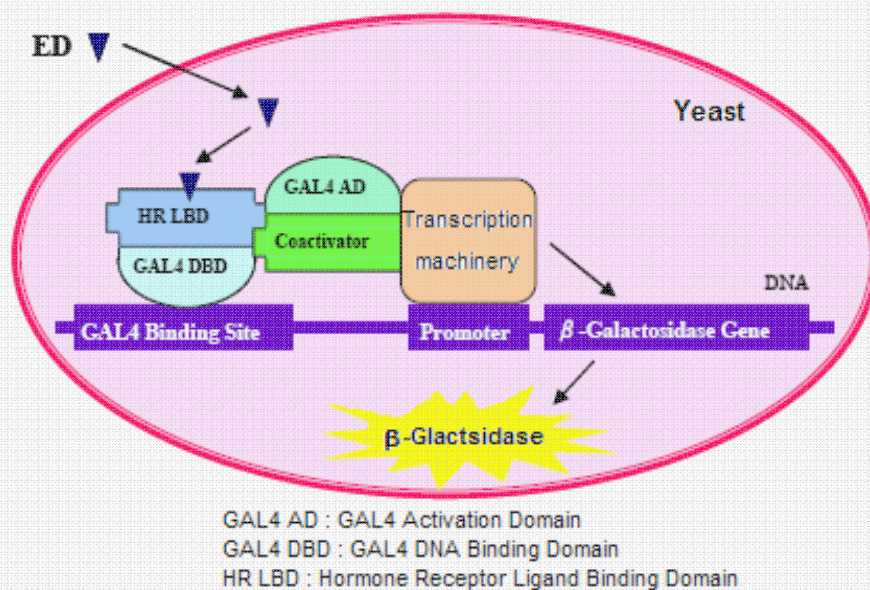
sensitive cells. The most commonly used is the **E-screen assay**, in which the proliferation of human MCF-7 breast cancer cells in response to estrogen is measured.

- **Reporter gene assays** are based on genetically engineered human cancer cells or yeast cells into which specific DNA sequences called estrogen response elements (ERE) have been added and linked to a reporter gene. Essentially, the assay works by quantifying the ability of a chemical to stimulate ER-dependent transcriptional activity. In this assay, reporter gene expression is the result of a cascade of molecular events following receptor activation, considered to provide a more integral indication of the estrogenic activity of a compound than competitive ligand binding or cell proliferation assays, e.g. YES (yeast estrogen screen) assay, and the **Yeast Two-Hybrid Assay** that is used in this project.
- **Enzyme-linked immunosorbent assays (ELISAs)** that depend on protein-receptor binding are commonly used for measurement of EDC residues, and can be obtained as commercial kits.

Yeast Two-Hybrid Assay

This assay is based on the ligand-dependent interaction of two proteins, a hormone receptor and a coactivator, and hormonal activity is detected by β-galactosidase activity.

Two expression plasmids, pGBT9-HRLBD and pGAAD424-TIF-2 are introduced into yeast cells, which carry a β-galactosidase reporter gene. Because the yeast strain harbours a GAL4 binding site upstream of a *lacZ* reporter gene, GAL4DBD-ER binds to



the regulatory region of the *lacZ* gene. If GAL4DBD-ER interacts with GAL4AD-coactivator, GAL4AD recruits the basal transcriptional machinery to the promoter region of the *lacZ* gene, resulting in production of β -galactosidase. Therefore, the β -galactosidase activity corresponds to the strength of interaction between ER and coactivator. The protein-protein interaction between ER and coactivator are strictly dependent on the presence of 17 β -estradiol.

Adapted from:

1. WHO/IPCS (2002). Global assessment of the state-of-the-science of endocrine disrupters. Edited by: Damstra T, Barlow S, Bergman A, Kavlock R, Van Der Kraak G. World Health Organisation / International Program on Chemical Safety.
2. Kinnberg K (2003). Evaluation of in vitro assays for determination of estrogenic activity in the environment (No. 43). Danish Environmental Protection Agency, Danish Ministry of the Environment.
3. Nishikawa J, Saito K, Goto J, Dakeyama F, Matsuo M, Nishihara T (1999). New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicology and Applied Pharmacology* 154:76-83.
4. Nishihara T, Nishikawa J, Kanayama T, Dakeyama F, Saito K, Imagawa M, Takatori S, Kitagawa Y, Shinjiro H, Utsumi H (2000). Estrogenic activities of 517 chemicals by yeast two-hybrid assay. *Journal of Health Science* 46:282-298.
5. Shiraishi F, Shiraishi H, Nishikawa J, Nishihara T, Morita M (2000). Development of a Simple Operational Estrogenicity Assay System using the Yeast Two-Hybrid System. *Journal of Environmental Chemistry* 10:57-64.

4. Results and Discussion

The primary aim of this research was to measure estrogenic and androgenic activities of treated municipal wastewater at *point of discharge* from WWTP to the wider environment. Another aim was to compare discharge activities and concentrations of specific hormones within and between the different WWTP groups to assess which, if any, treatment process and location (i.e. temperature) was most effective at reducing the estrogenic and androgenic activity in discharges.

- It is difficult to extrapolate results from one location (nation / state / region) to another (because of different project aims and methods used), although, where appropriate, we will make such comparisons to place our results in national and international context.

In total, there are ~185 WWTPs in Victoria (not including plants under construction, in process of being decommissioned, and those for which no information was available).

- Of these, 56% are in the north of the state (defined as being north of the Great Dividing Range), and 44% in the south.
- 124 (67%) are lagoon based plants, including those reporting treatment using maturation lagoons, facultative lagoons, oxidation ponds. Of these lagoon-based plants, two-thirds are in the north of the state.
- 61 (33%) are mechanical treatment plants, including those using various forms of activated sludge processes, extended aeration/flocculation technology, and trickle filters. Of these mechanical plants, two-thirds are in the south of the state.

The annual outflows of the forty-five WWTPs we surveyed in this study are as varied as that determined for Victoria's

WWTPs as a whole, i.e. there is a five order of magnitude difference in reported annual outflow between the smallest and largest plants (based on annual flows) (Figure 4.1).

- There is no difference in annual flow between the six treatment groups.

4.1 Androgenic activity and androgen concentrations

In the Winter 2006 survey, no sample produced a response in the androgen assay (hAR assay) (Figure 4.2). The results of the concurrent toxicity assay suggest that a lack of assay response was related to lack of androgenic compounds, rather than the direct toxic effect of the effluent, since most samples were non-toxic or weakly-toxic ($IC_{50} (C.R.) \geq 400$; Figure 4.3).

- In other words, lack of response by the hAR assay was not caused by the samples killing off the yeast, but rather suggested that either (a) androgenic activity was too low for the bioassay, and/or (b) the compounds are in fractions not studied.

That the lack of androgenic response is due to lack of androgens in the sample is in part assured because we measured androgenic activity in all three fractions (W, FL and RM), and found no activity in any fraction; and in part assured because the nature of the testing itself, i.e. the use of positive controls in the assay. In this case, the assay responded normally to the two positive controls used, namely dihydrotestosterone and 11-keto-testosterone.

That the sample preparation process does not remove steroid hormones from the sample was in part assured through early QA/QC procedures in which the hormones were tracked through the separation steps (*data not presented*), and thereafter the use of spiked composite WWTP effluent to

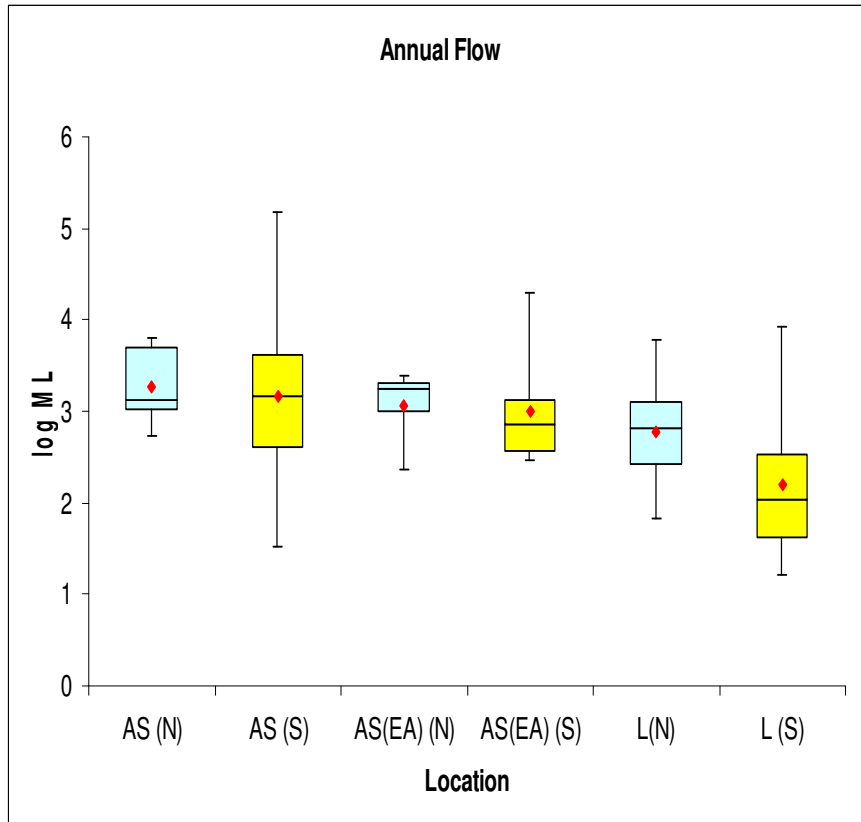


Figure 4.1 Summary of annual outflows for WWTP surveyed.

- Note: logarithmic scale; data as reported by water authorities directly to project team, or indirectly through other publications; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

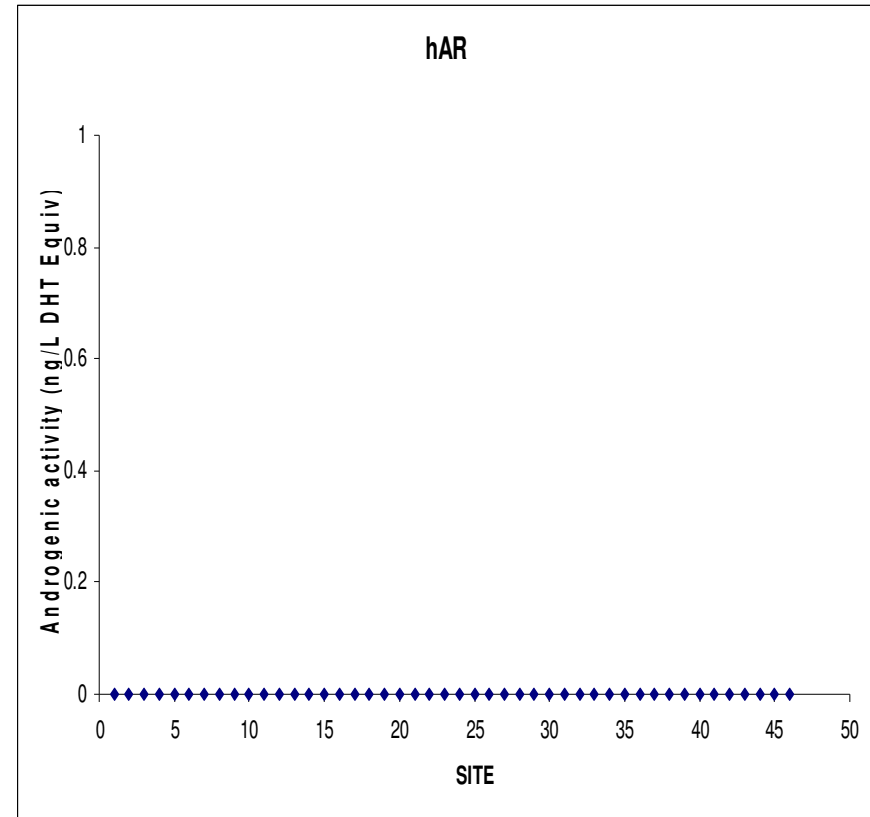


Figure 4.2 Summary of androgenic activity of Winter 2006 samples.

- As measured by hAR assay.

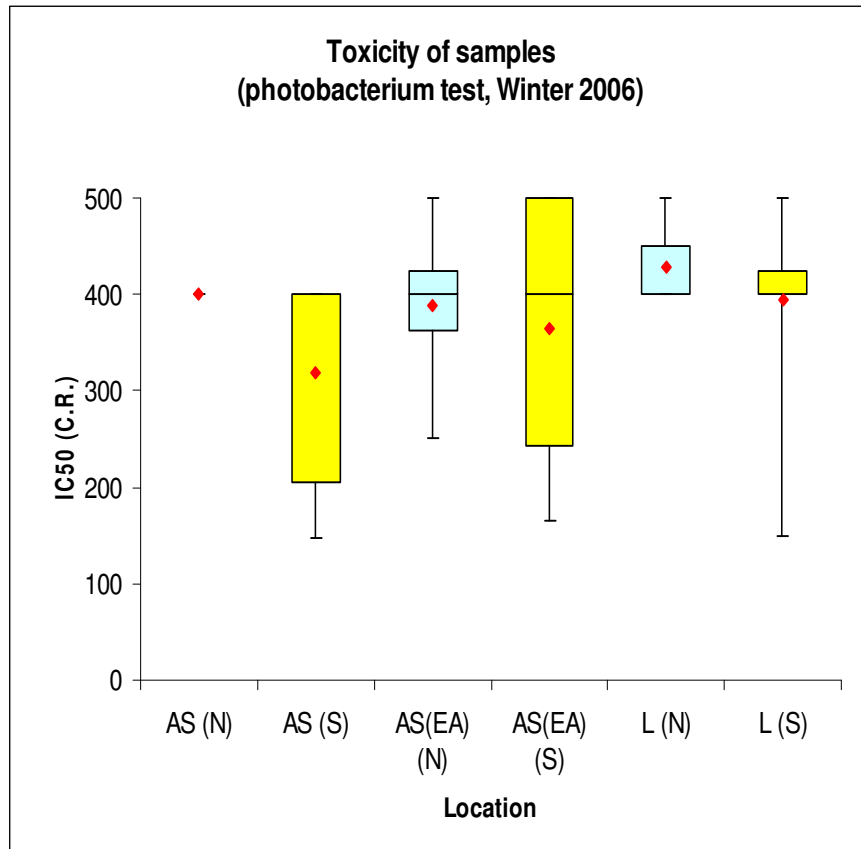


Figure 4.3 Summary of Winter 2006 sample toxicity.

- Note: the higher the IC₅₀ (C.R.), the lower the toxicity. Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

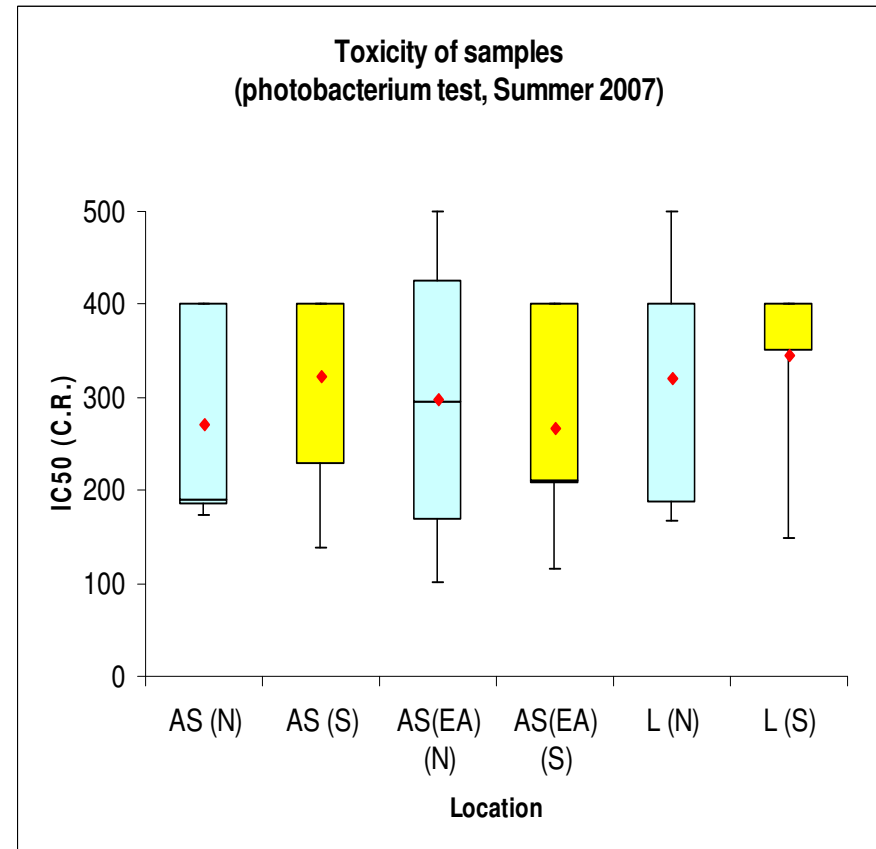


Figure 4.4 Summary of Summer 2007 sample toxicity.

- Note: the higher the IC₅₀ (C.R.), the lower the toxicity. Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

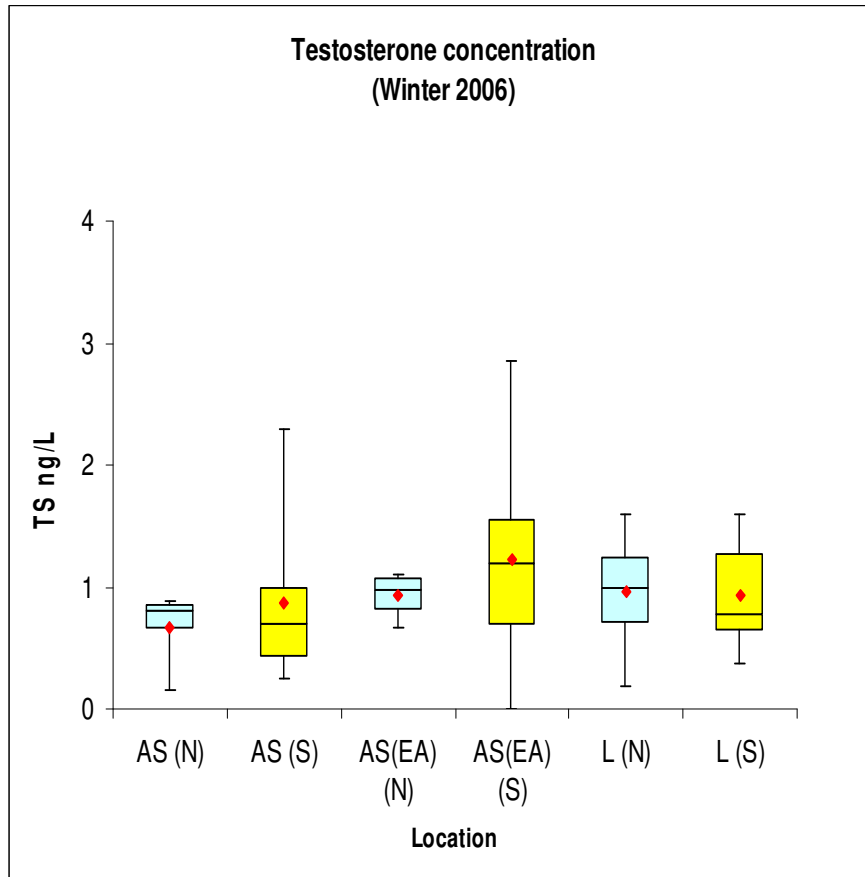


Figure 4.5 Summary of testosterone concentrations in Winter 2006 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

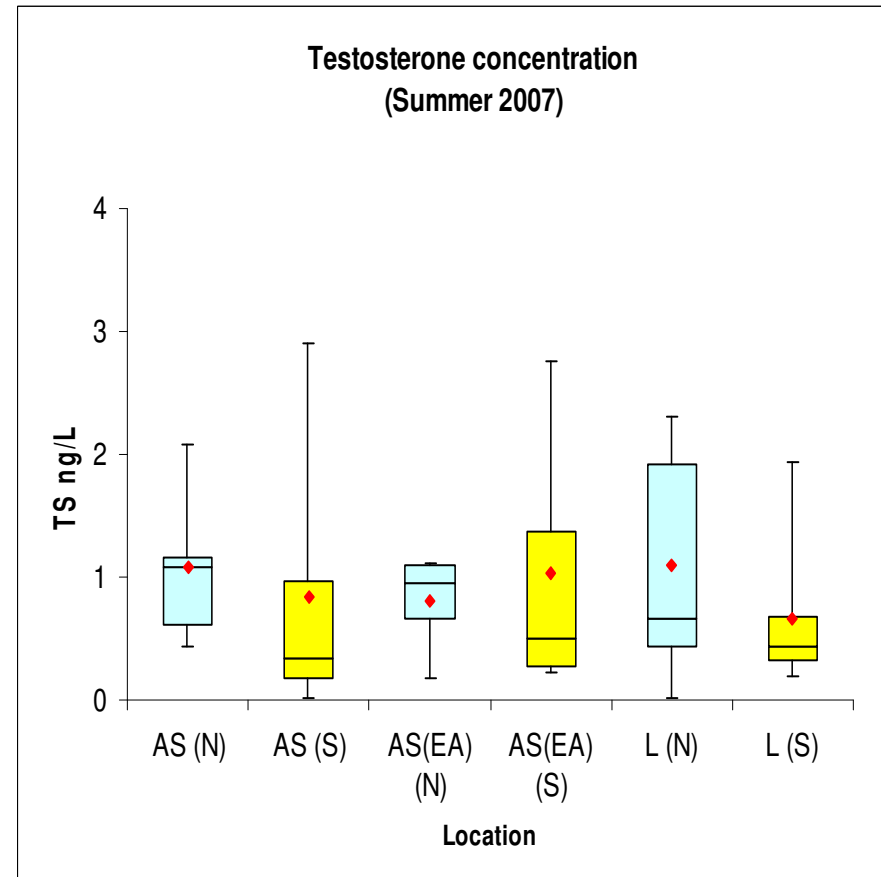


Figure 4.6 Summary of testosterone concentrations in Summer 2007 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

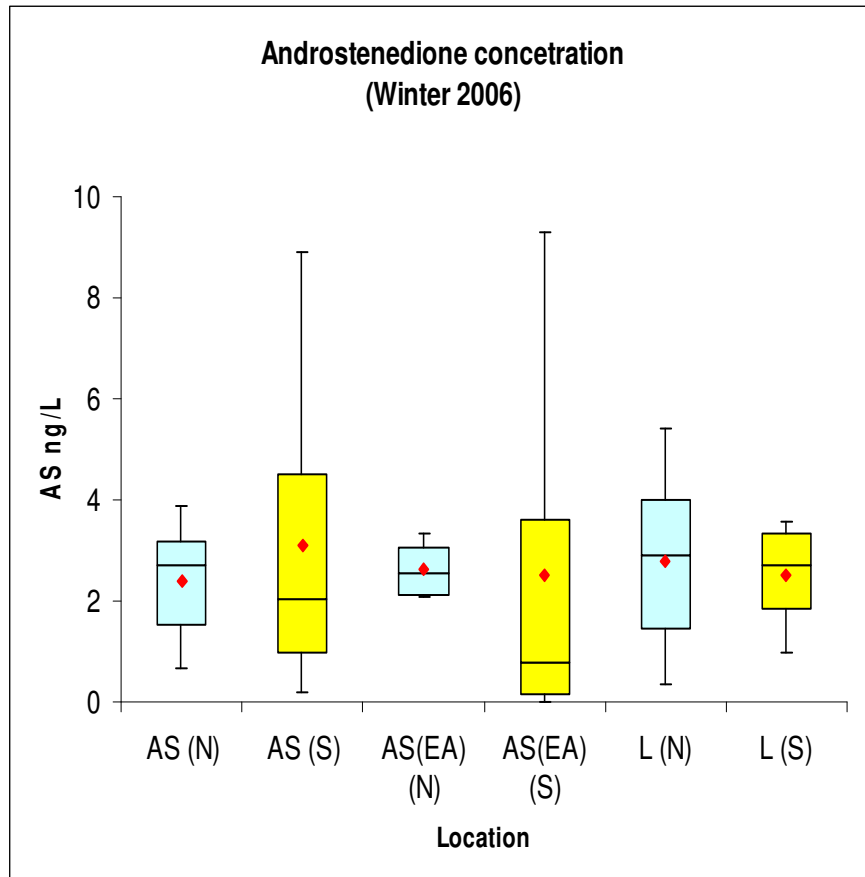


Figure 4.7 Summary of androstenedione concentrations in Winter 2006 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

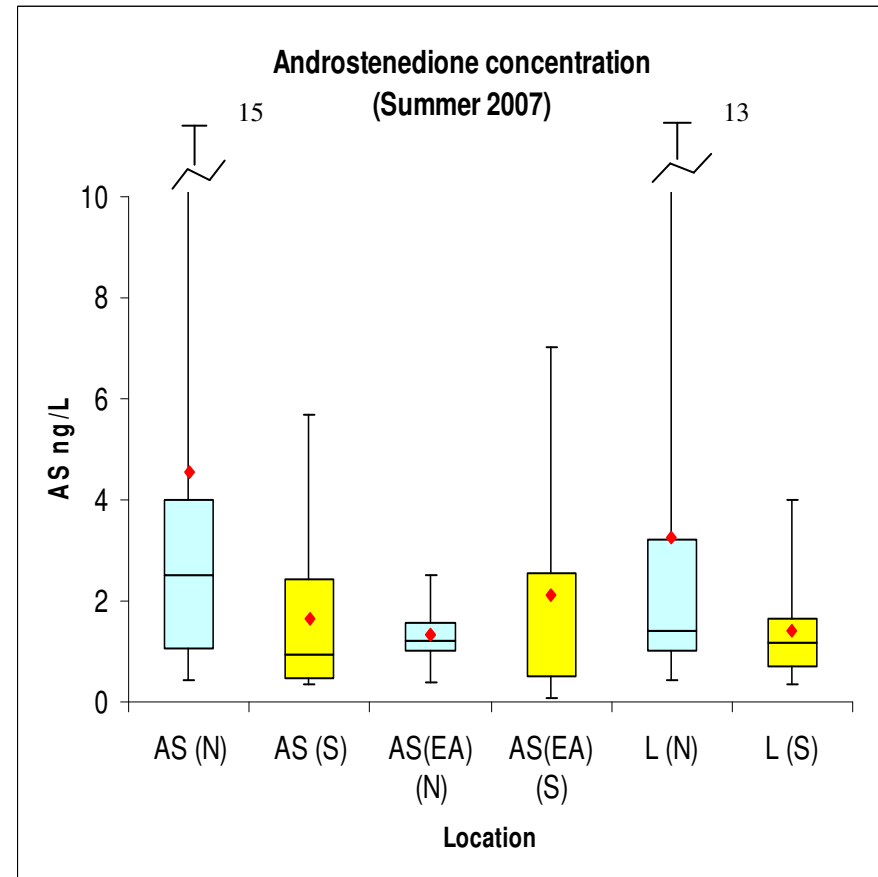


Figure 4.8. Summary of androstenedione concentrations in Summer 2007 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

assess analyte recovery (see Section 3.5.6). In this latter case, testosterone and androstenedione were quantitatively recovered in the FL fraction (if anything, the ELISA system overestimates testosterone and androstenedione concentrations).

- Because none of the Winter 2006 samples produced a response in the hAR assay, this test was not conducted on Summer 2007 samples.

Measurement of testosterone and androstenedione concentrations by ELISA suggests that there were indeed androgens present in both the Winter 2006 and Summer 2007 samples, but at concentrations generally below 3 ng/L (testosterone; Figure 4.5, 4.6) and below 10 ng/L (androstenedione; Figure 4.7, 4.8), and hence below the estimated limit of determination of the assay.

There are no statistical differences in the concentration of either testosterone or androstenedione within (i.e. north or south of state) or between treatments in either Winter 2006 or Summer 2007, and no statistical differences in the concentration of either androgen between Winter 2006 and Summer 2007.

The low levels of testosterone observed in our study are consistent with Fernandez et al.¹ and Esperanza et al.², neither of whom reported measurable amounts of testosterone in their surveys of Canadian and Spanish WWTPs, respectively.

The low levels of testosterone and androstenedione observed in our study are also broadly consistent with recent reports by Tan et al.³ of low ng/L concentrations of androsterone and etiocholanolone (metabolites of testosterone and androstenedione) in the effluent from five WWTPs in South East Queensland employing activated sludge as the

secondary biological treatment step (<BDL - ~ 26 ng/L).

- The rationale for choosing to monitor androsterone and etiocholanolone concentrations rather than testosterone and androstenedione is not explored by Tan et al.³, but it is perhaps not unreasonable to assume that low levels of these major metabolites in the effluents would have translated into low levels of parent compound in the effluents.

The lack of androgenic activity and the low levels of testosterone and androstenedione (or their metabolites) observed in our study is, however, somewhat inconsistent with reports by Leusch et al.⁴ of high, and highly variable, levels of androgenic activity in the final effluents of thirteen WWTPs in southern Queensland (12 suspended film/activated sludge plants, 1 trickle filter-based plant) and two WWTPs in Canterbury, New Zealand (one trickle filter-based plant, and one oxidation pond treatment system) (<6.5–736 ng/L testosterone equivalents).

- Chapman et al.²⁶ claim these levels are similar to those reported for WWTPs in the United Kingdom (< 113 - 4000 ng/L DHT equivalents in a yeast assay,⁵ and 34-635 ng/L (DHT equivalents in a yeast assay).⁶
- However, Kirk et al.⁵ actually report that androgenic activity was below their MDL (<113 ng/L DHT equiv.) in all bar one effluent, and the high levels of androgenic activity reported by Thomas et al.⁶ were for effluents receiving only primary treatment (cf. the suspended growth/activated sludge plants in Leusch et al.'s 2006 survey).⁴ Androgenic activity in the effluent from the activated sludge plants in Thomas et al.'s survey were < 23 ng/L DHT equiv.⁶

We must, however, be very careful not to make too much of these comparisons for a number of reasons, namely:

- The different target analytes and experimental protocols of the studies, including those associated with sample handling and preparation, and the different methods used to determine androgenic activity and hormone concentrations.
- That, nationally and internationally very little is known of androgenic activity and the levels of specific androgens in WWTP discharges (particularly cf. our knowledge of estrogens). Indeed, until recently, the only papers quoted when discussing androgenic activities in WWTP effluents were those of Kirk et al. ⁵ and Thomas et al. ⁶

In short, given the paucity of information available, nationally or internationally, any discrepancies between Victorian values and those in southern Queensland may not be real at all, rather the wide variability in hormonal activity observed (particularly in southern Queensland) may simply reflect reality – there is significant variation in androgenic activity and the concentration of specific androgens in WWTP effluents.

- More research needs to be done to verify the androgenic activity and specific hormone/metabolite levels reported in these studies.

Little is known about the effect of exposure of fish and other aquatic organisms to androgenic chemicals originating from WWTPs, although some studies have shown masculinization of mosquito fish exposed to paper mill effluents containing ng/L levels of the steroid androstenedione ^{7,8}.

- More research needs to be done to determine if the androgenic activity reported in recent Australian studies is

sufficient to induce a physiological effect in exposed fish.

4.2 Estrogenic activity and estrogen concentrations

Almost all of the effluents examined in both the Winter 2006 and Summer 2007 surveys showed estrogenic activity, to a greater or lesser extent, in both the hER and mER assays (Figures 4.9 - 4.12).

- Winter 2006
 - hER, not detected – 7.9 ng/L EEQ;
 - mER, not detected – 11 ng/L EEQ (with one sample 61.5 ng/L EEQ).
- Summer 2007
 - hER, not detected – 16 ng/L EEQ;
 - mER, not detected – 18 ng/L EEQ.

In both Winter 2006 and Summer 2007, estrogenic activity is correlated with sample toxicity (much more strongly so in Summer 2007), i.e. low activity, high toxicity.

- Consequently, in discussing the levels of estrogenic activity observed, we must consider that a lack of assay response, or a low response, may be related to the direct toxic effect of the effluent, i.e. no/low response because the yeast is being adversely affected by toxic components in the sample.

There are no statistical differences in estrogenic activity within (i.e. north or south of state) or between treatments in samples collected in the Winter 2006 survey. There are also no statistical differences in estrogenic activity within treatments (i.e. between samples collected from the north of the state and those collected from the south of the state) in the Summer 2007 survey, regardless of treatment type. However, there was a statistically significant difference in estrogenic activity between activated sludge-based (AS) plants and

Table 4.1 A comparison of estrogenic activity reported for Victorian wastewater treatment plant effluents with that reported elsewhere in Australia and New Zealand and internationally studies.

Location	Method	Concentration (ng/L EEQ)	Reference
Canada	YES	N.D. - 96	Servos et al., 2005 ⁹
China	Recombinant yeast assay	0.05 – 0.5	Ma et al., 2007 ¹⁰
Finland	Bioluminescent yeast assay	4 - 7	Salste et al., 2007 ¹¹
Germany		< 1	Andersen et al., 2003 ¹²
Germany	YES	65.9 ± 10.4	Pawlowski et al., 2004 ¹³
Japan	Yeast assay	5 - 15	Matsui et al., 2000 ¹⁴
Sweden	YES	<0.1-15	Svenson et al., 2003 ¹⁵
Switzerland	YES	0.1-90	Rutishauser et al., 2004 ¹⁶
USA	YES	44-151	Tilton et al. 2002 ¹⁷
USA	E-screen	1- 2	Shappell, 2006 ¹⁸
Australasia			
Northern Territory	YES	0.098 ± 0.01	Hogan et al., 2005 ¹⁹
Western Victoria	Y2h ^a	N.D. - 55	Mispagel et al., 2005 ²⁰
Western Victoria	Y2h ^b	N.D. - 42	Mispagel et al., 2005 ²⁰
Southern Queensland	ER ^c	< 0.75	Leusch, 2005 ²¹
Southern Queensland, New Zealand	ER ^c	<1 - 4.2	Leusch, 2006 ⁴
Southern Queensland	E-screen	<1 - 67.8	Tan et al., 2007 ³
Southern Victoria	Y2h ^a	<0.5 - 45	Allinson, unpublished
Southern Victoria	Y2h ^b	<0.5 – 7.9	Allinson, unpublished
New Zealand	ER ^d	50	Bandelj et al., 2006 ²²
Victoria (winter)	Y2h ^a	N.D. - 61.5	This study
Victoria (summer)	Y2h ^a	N.D. - 18	This study

- EEQ, estradiol equivalents; a, yeast two hybrid assay incorporating Japanese medaka estrogen receptor; b, yeast two hybrid assay incorporating human estrogen receptor; c, estrogen receptor binding assay utilising receptors isolated from sheep; d, estrogen receptor binding assay utilising receptors isolated from rainbow trout liver; N.D., not detected.

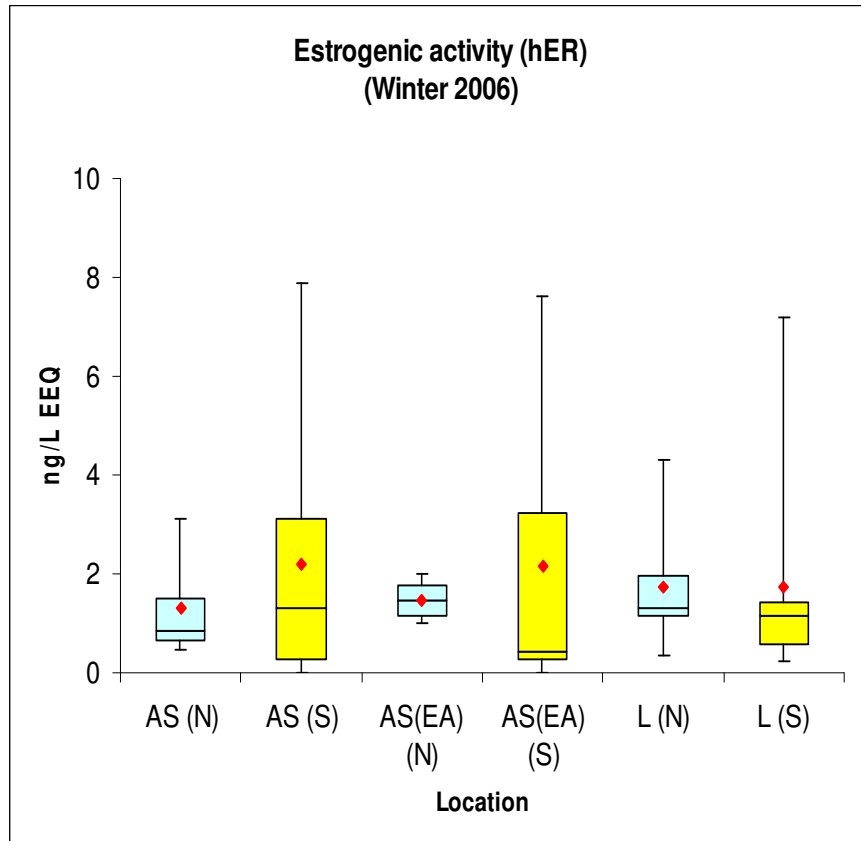


Figure 4.9 Summary of estrogenic activity (hER) in Winter 2006 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

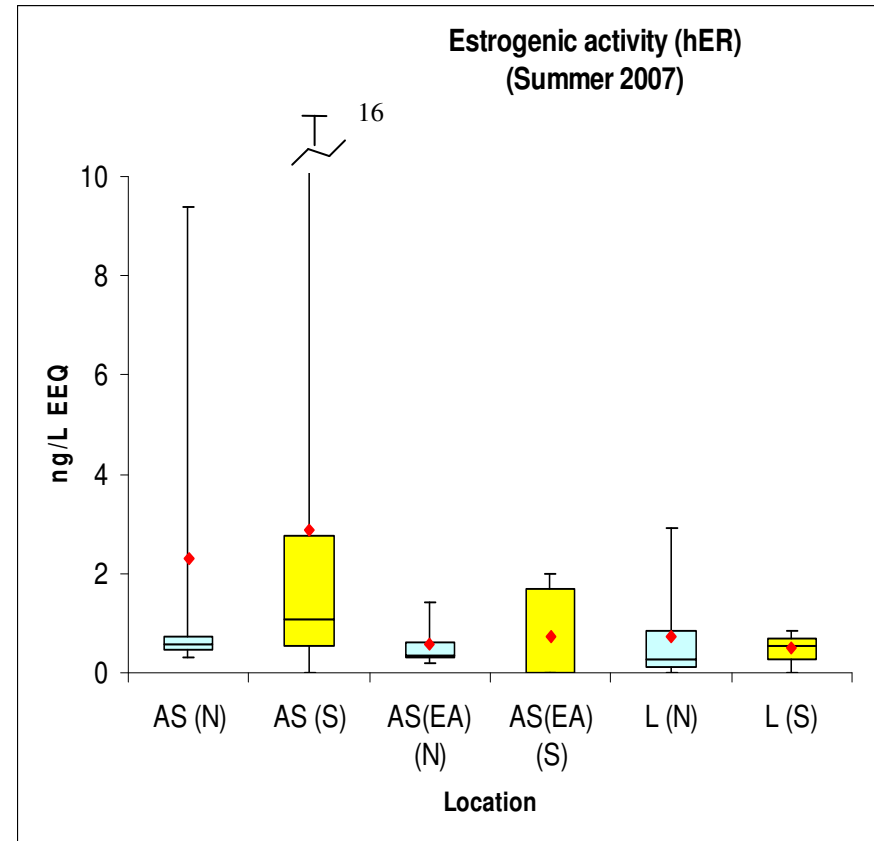


Figure 4.10 Summary of estrogenic activity (hER) in Summer 2007 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

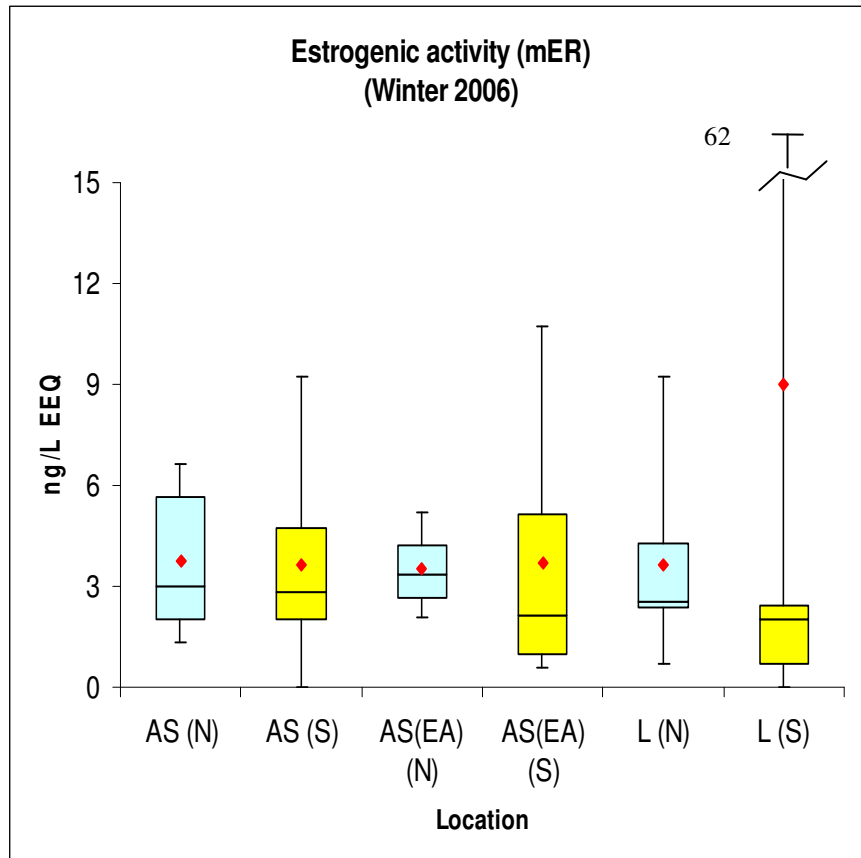


Figure 4.11 Summary of estrogenic activity (mER) in Winter 2006 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

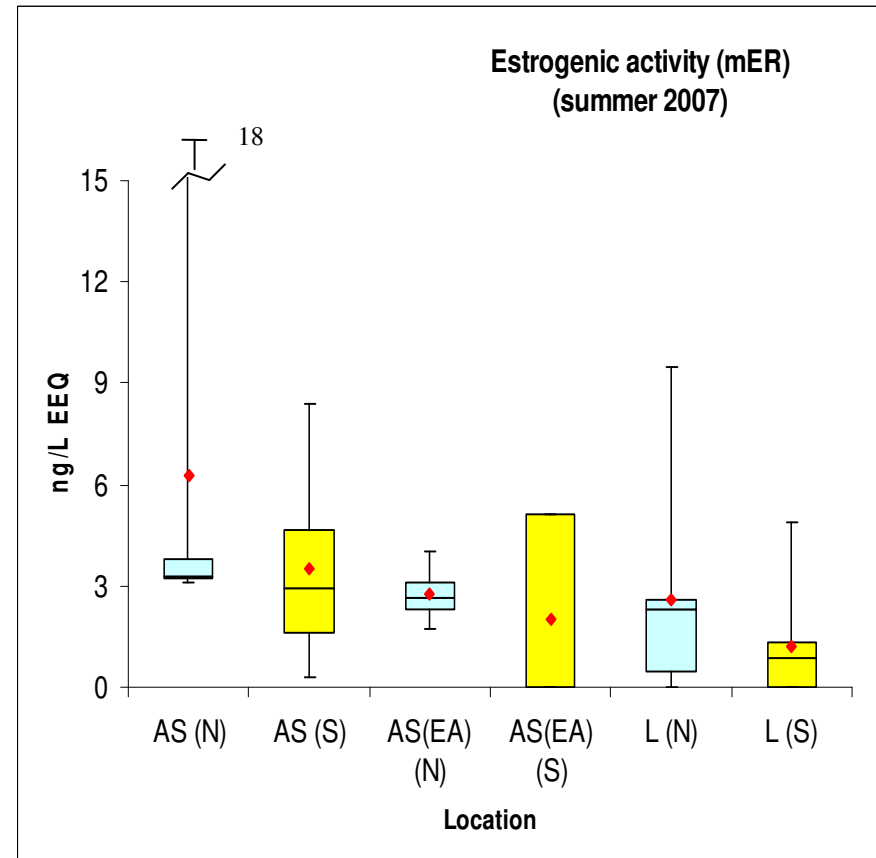


Figure 4.12 Summary of estrogenic activity (mER) in Summer 2007 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

lagoon-based (L) WWTPs in Summer 2007 (mER assay, $p < 0.05$). There was also a statistically significant difference in estrogenic activity between Winter 2006 and Summer 2007, with higher activity in Winter 2006 ($p < 0.05$).

In both Winter 2006 and Summer 2007, hER and mER estrogenic activity is very strongly correlated. In other words, although the estrogenic activity as measured by hER is generally lower than that measured by mER, where there is high hER activity there is also high mER activity.

- The hER assay is more sensitive to estradiol (and estradiol-related) compounds than the mER assay. On the other hand, the mER assay is somewhat more sensitive to xenoestrogens such as alkylphenols.

The estrogenic activity observed in this study is comparable with that of Mispagel et al. ²⁰, and that recently reported by Tan et al. ³ in Australia and New Zealand (Table 4.1). In the latter case, the estrogenic activity of effluent from five municipal wastewater treatment plants (WWTPs) in South East Queensland, Australia was determined using E-Screen assays at between $< 1 - 14.8$ ng/L EEQ (with the exception of one effluent sample which was at 67.8 ng/L EEQ).

On the whole, the levels of estrogenic activity observed in this study were consistent with that observed in the northern hemisphere, e.g. in Japan (5-15 ng/L EEQ) ¹² the USA (21-147 ng/L EEQ) ¹⁵ Sweden ($< 0.1 - 15$ EEQ) ¹³ and Switzerland ($< 1 - 90$ ng/L EEQ) ¹⁴ respectively (Table 4.1).

Estradiol and estrone were observed in almost all of the effluents examined in both the Winter 2006 and Summer 2007 surveys, to a greater or lesser extent (Figures 4.13 - 4.16).

- Winter 2006

- estradiol, $< 0.1 - 12.4$ ng/L.
- estrone, $< 0.2 - 16.8$ ng/L.

- Summer 2007

- estradiol, $< 0.1 - 18.5$ ng/L.
- estrone, $< 0.1 - 32.0$ ng/L.

There are no statistical differences in estradiol or estrone concentrations within (i.e. north or south of state) or between treatments in samples collected in the Winter 2006 survey. There are also no statistical differences in estrone concentration within treatments (i.e. between samples collected from the north of the state and those collected from the south of the state) in the Summer 2007 survey, regardless of treatment type. However, in the Summer 2007 survey, there was a statistically significant difference in estradiol concentration within treatments for the extended aeration (AS(EA)) and lagoon (L) groups ($p < 0.05$). For both estradiol and estrone, there was a statistically significant difference in concentration between treatments for the activated sludge-based (AS) plants and lagoon-based (L) plants ($p < 0.05$). Finally, estradiol and estrone concentrations were higher in the Winter 2006 survey than in the Summer 2007 survey ($p < 0.05$), particularly in the lagoon-based WWTP effluents.

The estradiol and estrone concentrations observed in this study are comparable with those seen elsewhere in Australia and New Zealand (e.g. ^{23,24}), and at the lower end of the range of concentrations reported internationally (Table 4.2, 4.3), particularly for estrone.

Estrogens, such as estradiol, are excreted by humans as inactive glucuronide or sulfonate conjugates. Microbial activity in the sewerage system and treatment plants results in deconjugation of these compounds. Deconjugation causes the hormones to resume their active form.

- The higher estrogenic activity / estrogen concentrations seen in the Winter survey may have been, in part, caused by deconjugation during water storage in lagoons (including those associated with many activated sludge-based WWTPs) leading to an increase in measured estrogenic activity/estrogen concentration in the discharged water.
- The lower estrogenic activity / estrogen concentrations seen in the Summer 2007 survey may have been, in part, caused by much shorter retention times in the WWTP systems, in and of itself caused by the off-site demand for the water (e.g. by irrigators).
- Servoc et al. ¹⁰ noted that, while there was no statistical difference, the lagoons that had extremely long hydraulic retention time (HRT) and solids retention time (SRT) had consistently high removal of estrogens and estrogenic activity.
- The current study used a limited number of grab samples of the final effluent (environmental discharge) of many different plants with a wide variety of treatment and process characteristics. Additional, controlled studies are needed to more fully explore the potential relationship between extended HRT and SRT on removal of estrogens.

In its early stages, the project was faced with a choice, whether to conduct in-depth investigations on a limited number of WWTPs (i.e. from raw influent to treated effluent), or to take a broader look at a number of WWTPs (i.e. measure hormonal activity at a large number of locations). After much discussion, the Project Steering Committee decided to take the latter approach, in part justifying the decision on the paucity of information on hormonal activity of Victorian WWTP discharges. Consequently, we did not collect raw

wastewater samples, or samples through treatment trains, and we are thus unable to determine the estrogen and androgen removal efficiencies of the WWTP investigated.

Although there have been few detailed comparative studies, secondary treatment using suspended solids processes is considered more effective at reducing the estrogenic activity in wastewater than biological filter-based treatment ²⁵, removing more than 90% of the activity in primary-treated wastewater. Leusch et al. ^{21, 4} suggest activated sludge treatment is particularly effective, removing >99% of the estrogenic activity in the raw wastewater. Braga et al. ²³ suggest lower removal efficiencies, but still report activated sludge treatment removes more than 85% of E1 and E2.

The removal efficacy of biological filter-based treatment plants, such as trickling filters, appears to be much more variable. Chapman et al. ²⁶ report a trickling filter WWTP in southern Queensland removed 92% of the estrogenic activity, while a New Zealand trickling filter WWTP actually caused an increase in estrogenic activity. Johnson et al. ²⁵ suggested that removal efficiencies of 10 biological filter plants in the U.K. were nearer 30% (for estrogenic activity).

- While treatment efficiency of activated sludge plants may be high, if the hormonal activity of the raw wastewater is high, then discharge activity may still have unacceptably high levels of hormonal activity (from an aquatic risk perspective).
- From an environmental risk assessment perspective, the highest priority is not to know how good a particular plant, or type of system is at reducing the levels of a contaminant, but rather to know the levels in the waters that are discharged to the environment.

Chapman et al. ²⁶ suggest that the poor efficacy of the New Zealand trickling filter plant (cf. the plant in southern Queensland) may be related to the lower ambient air temperature (4-14°C at the New Zealand plant compared with 14-21°C at the Queensland plant). Chapman et al. ²⁶ also suggest that the slightly higher estrogenic activity seen in effluents in New South Wales and Victoria (cf. southern Queensland) is also likely due to the colder climate in the southern states (resulting in lower removal efficiency of estrogenic chemicals).

The recent results by Tan et al ³ (<1–67.8 ng/L EEQ; Table 4.1) in part negate the hypothesis put forward by Chapman et al. ²⁶ because they indicate that estrogenic activity in southern Queensland may, in fact, not be much different from that observed in Victoria. However, Tan et al's results were unavailable when we began this study, so to address the temperature hypothesis, in this study we collected ambient temperature information for the nearest weather stations to our sampling sites for the months in which our samples were collected.

- In August 2006, the southern WWTPs were, on average, several degrees warmer than their counterparts in the north of the state, yet there was no difference in hormonal activity, or hormone concentrations within treatments (north cf. south).
- In February/March 2007, the northern WWTPs were, on average, some 12 degrees warmer than their counterparts in the south of the state, yet hormonal activity, and hormone concentrations within treatments were significantly different (north higher than south).
- The results suggest that ambient temperature may have some influence on hormonal activity of the WWTP discharges studied, but that ambient

temperature is only one of a number of potential influences, and indeed may not be an adequate surrogate for actual water temperature in the WWTP systems. Further work assessing the influence of actual effluent temperature on hormonal activity is required.

4.3 Potential Environmental Risk

The list of trace contaminants found in wastewater is long. However, in general, natural hormones (e.g. E1, E2, and E3) are considered the major contributors to the estrogenic activity observed in most sewage effluents, and subsequently in the receiving water. In the northern hemisphere, fish exposed to WWTP effluent (or wastewater contaminated water in rivers) have been found to exhibit a wide array of biochemical and physiological effects. Of these, perhaps the most often quoted are abnormally high levels of vitellogenin (Vtg) in males, and high incidences of intersexuality in gonochoristic species ²⁷.

- Vitellogenin is a protein synthesized by oviparous vertebrates under estrogenic stimulation ²⁸, and hence normally only produced in significant quantities in females. Exposure to xenoestrogens can induce Vtg production in males ²⁹, although it is unclear if high levels of Vtg in males have reproductive impacts.
- Intersex in fish is primarily characterized by the presence of both male and female sex cells in the gonads of an individual (ovotestis).

The risk to fish of exposure to environmental estrogens has been demonstrated in both the field and laboratory.

- Jobling et al. ³⁰ found a high incidence of intersexuality in wild populations of roach in eight rivers in the UK,

especially those fish captured downstream of WWTP outfalls. Similar findings have been reported in Danish streams receiving WWTP effluent ³¹.

Such intersex has been shown to be caused in a dose-dependent manner ³².

- Male mosquito fish (*Gambusia holbrooki*) collected in wastewater-contaminated water in New South Wales have shown evidence of feminization of secondary sexual characteristics consistent with exposure to estrogenic chemicals ³³.

One of the main aims of this study was to provide water authorities with an understanding of the range of estrogenic activity in Victorian WWTP effluents, and a consideration of potential impact in receiving waters. From an aquatic environmental perspective, one might consider it appropriate to look at the data produced by the assay incorporating a fish estrogen receptor (mER assay), particularly if the intention is to protect fish and other organisms in receiving waters. However, although such *in vitro* assays are useful as screening tools for monitoring studies, they are a simplification of the *in vivo* situation, i.e. although they are living systems *in vitro* tests are, to put it simply, not fish, and do not address effects that result from multiple mechanisms or take into account processes such as bioavailability, toxicokinetics, metabolism or cross-talk between biological pathways. Unless, and until, we can address the latter using *in vivo* tests, risk can only partially be addressed by comparing specific chemical concentrations in the effluent with classical toxicological experiments. In this case, estradiol is the most appropriate chemical to use.

- The lowest level of E2 at which physiological effects have been observed in fish (rainbow trout; reduced semen volume, sperm density

and sperm fertility) is 1 ng/L.

(Lahnsteiner et al., 2006; Table 4.4) ³⁴.

- The next lowest level at which physiological effects (intersex) have been observed in fish (medaka) is 8.7 ng/L E2 (Seki et al., 2005) ³⁵.
- The lowest level at which induction of Vtg has been observed in laboratory studies using fish (trout) is 1 ng/L E2 (Purdom et al., 1994; Hansen et al., 1998; Table 4.5) ^{36, 29}.

Apart from some of the southern lagoon systems, for the most part estradiol concentrations were above 1 ng/L E2 and below 10 ng/L E2. One assumption commonly made is that WWTP discharges will be diluted significantly in the receiving environment. This may not be appropriate in some circumstances, e.g. where the discharge represents all, or most of, the environmental flow in a waterway, or where discharges are to enclosed water bodies (e.g. lakes). In such cases, there may be significant risks to aquatic wildlife.

- The levels of estrogenic activity seen in our Victorian WWTPs are likely to be an environmental risk if WWTP discharge represents a major component of stream flow.
- In this broad-brush and simplistic assessment of risk, no account is taken of the potential of low concentrations of highly active compounds (such as ethinyl estradiol) to increase the perceived risk of the effluents, or of the additive effects of mixtures of chemicals, i.e. the chemical cocktail found in the effluent may induce effects equivalent to the sum of the effects seen by individual chemicals in the mixture at equivalent concentrations.

In short, this study provides some reassurance that, for the most part, Victorian environments are probably not at risk from the steroid hormones in WWTP effluents (at least, acting on their own), but

Table 4.2 A comparison of estradiol concentrations in Victorian wastewater treatment plant effluents with that reported elsewhere in Australasia and internationally.

Location	Method	Concentration (ng/L)	Reference
Canada	GC/MS/MS	N.D. – 64	Ternes et al., 1999 ³⁷
Canada	GC/MS	N.D. – 13.9	Lishman et al., 2006 ³⁸
Canada	GC/MS	~3 - ~15 *	Fernandez et al., 2007 ¹
Canada		0.2 – 14.7	Servos et al., 2005 ⁹
Denmark		<1 – 4.5	Andersen et al., 2004 ³⁹
France	GC/MS	4.5 – 8.6	Cargouët et al., 2004 ⁴⁰
Germany	GC/MS/MS	N.D. – 3	Ternes et al., 1999 ³⁷
Germany	GC/MS	<0.1 – 15	Kuch & Ballschmiter, 2001 ⁴¹
Germany	GC/MS	< 1 ng/L	Andersen et al. 2003 ¹²
Germany		<0.5 – 1.5	Hansen et al., 1998 ²⁹
Germany	ELISA	3.1 - 51	Hintemann et al., 2006 ⁴²
Germany	GC/MS/MS	<0.5 - 1	Joss et al., 2004 ⁴³
Italy	LC/MS/MS	N.D. - 7	Johnson et al., 2000 ⁴⁴
Italy	LC/ESI/MS/MS	0.35 - 35	Baronti et al., 2000 ⁴⁵
Japan	LC/MS/MS	0.3 – 2.5	Isobe et al., 2003 ⁴⁶
Japan	LC/MS/MS	N.D. - 11	Komori et al., 2004 ⁴⁷
Japan	ELISA	7 - 9	Matsui et al., 2000 ¹⁴
Japan	GC/MS	~0.5 - ~15 *	Nakada et al., 2006 ⁴⁸
Netherlands	GC/MS/MS	< 0.6 - 12	Belfroid et al., 1999 ⁴⁹
Netherlands	LC/MS/MS	N.D. - 12	Johnson et al., 2000 ⁴⁴
Spain	GC/MS/MS	<1	Carballa et al., 2004 ⁵⁰
Sweden	GC/MS	1.1	Larsson et al., 1999 ⁵¹
Switzerland	GC/MS	<0.5–6.4	Rutishauser et al., 2004 ¹⁶

- *, calculated from author's manuscript; N.D., not detected.

Table 4.2 (cont^d)

Location	Method	Concentration (ng/L)	Reference
Taiwan	LC/MS/MS	N.D. – 44.5	Chen et al., 2007 ⁵²
U.K.	GC/MS	2.7 - 48	Desbrow et al., 1998 ⁵³
U.K.	GC/MS/MS	<0.3	Fawell et al., 2001 ⁵⁴
U.K.	GC/NCI/MS	1.6-7.4	Xiao et al., 2001 ⁵⁵
U.K.		4.0 - 48	Hansen et al., 1998 ²⁹
USA	GC/NCI/MS	< 1 - 6	Drewes et al., 2005 ⁵⁶
USA	ELISA	0.6 – 6.2	Drewes et al., 2005 ⁵⁶
USA	GC/MS	1.0 ± 0.4	Esperanza et al., 2007 ²
USA	LC/MS/MS	0.2- 0.26	Reddy et al., 2005 ⁵⁷
Australasia			
Southern Queensland	-	<5	Chapman, 2003 ⁵⁸
Southern Queensland	GC/MS	4.7	Khan, 2004 ⁵⁹
New South Wales	GC/MS	BDL - 12	Braga, 2005a, 2005b ^{23, 60}
Queensland	E-Screen	<0.75	Leusch, 2005 ²¹
New Zealand	GC/MS	Trace - 14.8	Sarmah, 2006 ²⁴
Vic (winter)	ELISA	N.D. – 12.4	This study
Vic (summer)	ELISA	N.D. – 18.5	This study

Table 4.3 A comparison of estrone concentrations in Victorian wastewater treatment plant effluents with that elsewhere in Australasia and internationally.

Location	Method	Concentration (ng/L)	Reference
Canada	GC/MS/MS	N.D. – 48	Ternes et al., 1999 ³⁷
Canada	GC/MS	16 - 49	Lishman et al., 2006 ³⁸
Canada		1–96	Servos et al., 2005 ⁹
Denmark		<2 - 11	Andersen et al., 2004 ³⁹
France	GC/MS	6.2 – 7.2	Cargouët et al., 2004 ⁴⁰
Germany	GC/MS	< 1 ng/L	Andersen et al., 2003 ¹²
Germany	GC/MS/MS	N.D. – 70	Ternes et al., 1999 ³⁷
Germany	GC/MS	<0.1 – 15	Kuch & Ballschmiter, 2001 ⁴¹
Germany		<1	Hansen et al., 1998 ²⁹
Germany	GC/MS/MS	<0.5 – 8.6	Joss et al., 2004 ⁴³
Italy	LC/MS/MS	N.D. - 7	Johnson et al., 2000 ⁴⁴
Italy	LC/ESI/MS/MS	2.5 - 82	Baronti et al., 2000 ⁴⁵
Japan	LC/MS/MS	N.D. - 180	Komori et al., 2004 ⁴⁷
Japan	GC/MS	~3 - ~100 *	Nakada et al., 2006 ⁴⁸
Japan	GC/MS	5.9 - 60	Nakamura et al., 2007 ⁶¹
Netherlands	GC/MS/MS	< 0.4 - 47	Belfroid et al., 1999 ⁴⁹
Netherlands	LC/MS/MS	<0.4 - 47	Johnson et al., 2000 ⁴⁴
Spain	GC/MS/MS	4.4	Carballa et al., 2004 ⁵⁰
Sweden	GC/MS	1.1	Larsson et al., 1999 ⁵¹
Switzerland	GC/MS	4–50.5	Rutishauser et al., 2004 ¹⁶
Taiwan	LC/MS/MS	N.D. – 48.6	Chen et al., 2007 ⁵²
U.K.	GC/MS	1.4 - 76	Desbrow et al., 1998 ⁵³
U.K.	GC/MS/MS	<0.3	Fawell et al., 2001 ⁵⁴

• *, calculated from manuscript data; N.D., not detected.

Table 4.3 (cont^d)

Location	Method	Concentration (ng/L)	Reference
U.K.	GC/NCI/MS	6.4 - 29	Xiao et al., 2001 ⁵⁵
U.K.		1.4 - 76	Hansen et al., 1998 ²⁹
USA	GC/NCI/MS	0.6 – 50.4	Drewes et al., 2005 ⁵⁶
USA	GC/MS	9.0 ± 0.7	Esperanza et al., 2007 ²
USA	LC/MS/MS	0.7- 0.72	Reddy et al., 2005 ⁵⁷
Australasia			
Southern Qld	-	<13	Chapman, 2003 ⁵⁸
NSW	GC/ECD, HPLC, ELISA	13 - 430	Li, 2004 ⁶²
Qld	GC/MS	92	Khan, 2004 ⁵⁹
NSW	GC/MS	BDL - 54	Braga, 2005a, 2005b ^{23, 60}
New Zealand	GC/MS	Trace - 84.7	Sarmah, 2006 ²⁴
Vic (statewide, winter)	ELISA	N.D. – 16.8	This study
Vic (statewide, summer)	ELISA	N.D. – 32.0	This study

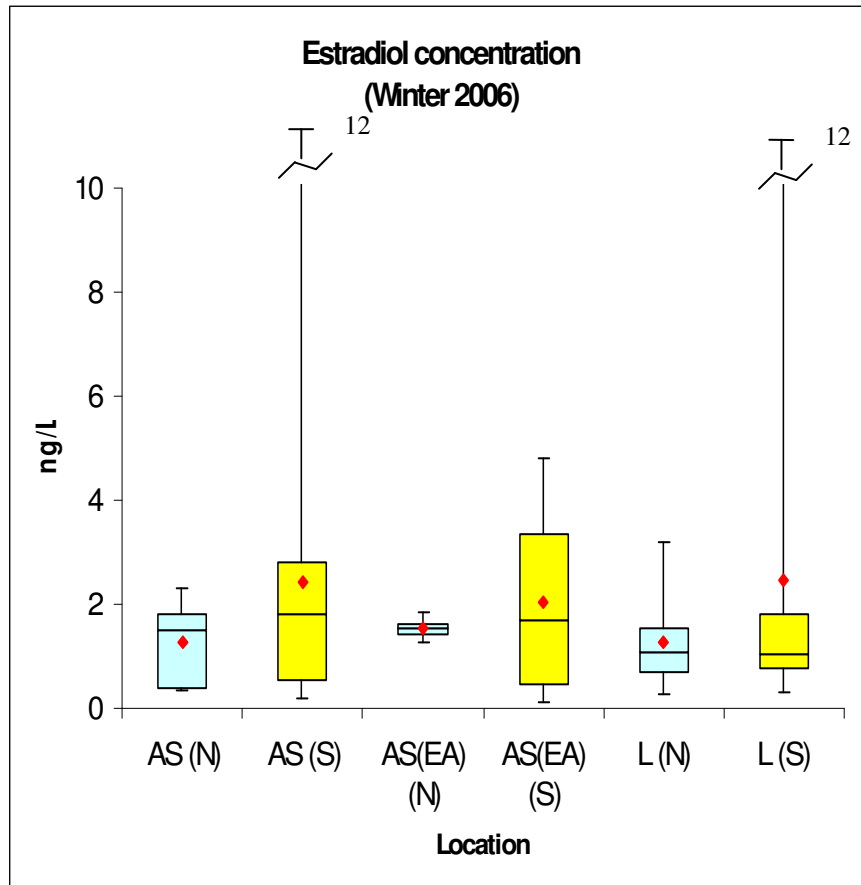


Figure 4.13 Summary of estradiol concentrations in Winter 2006 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

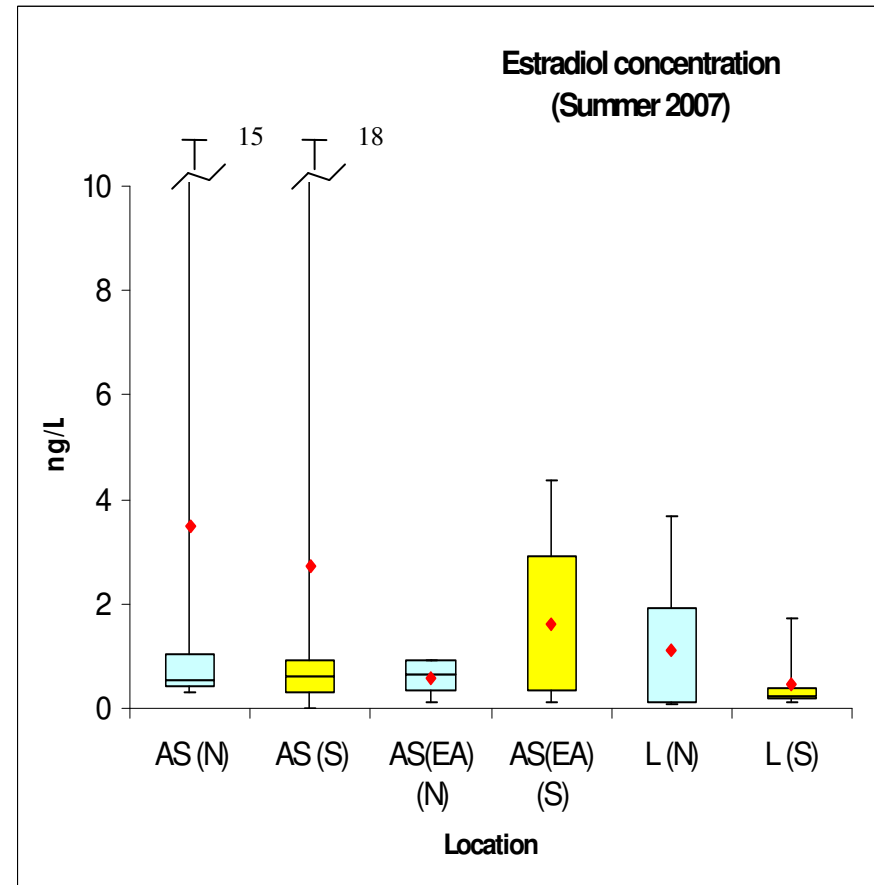


Figure 4.14 Summary of estradiol concentrations in Summer 2007 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

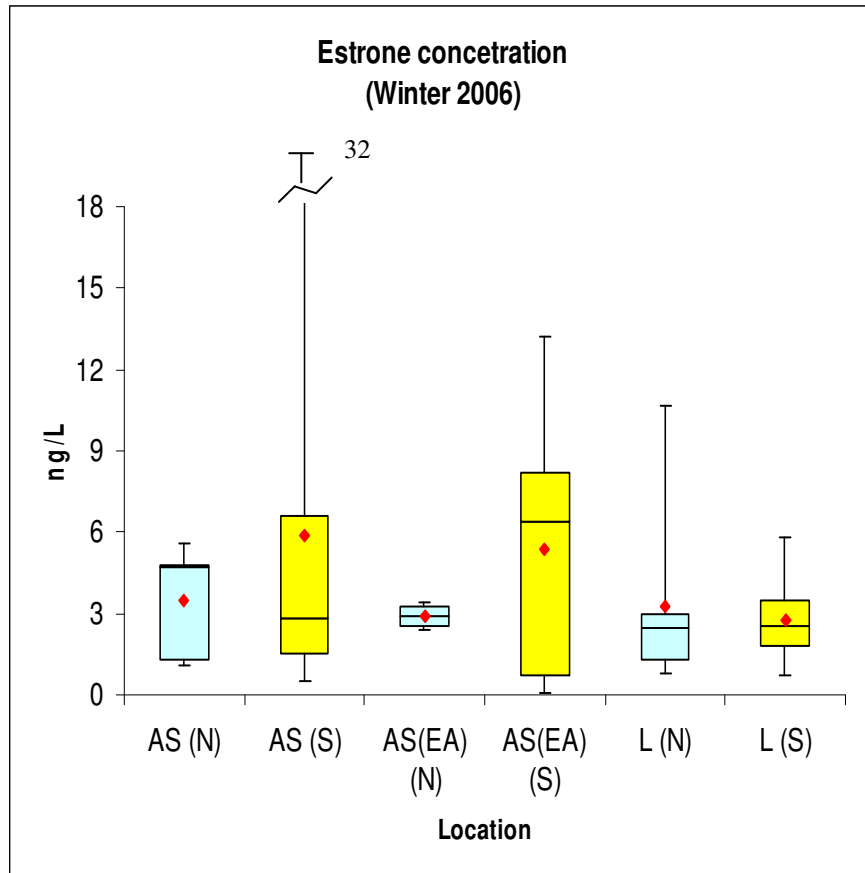


Figure 4.15 Summary of estrone concentrations in Winter 2006 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

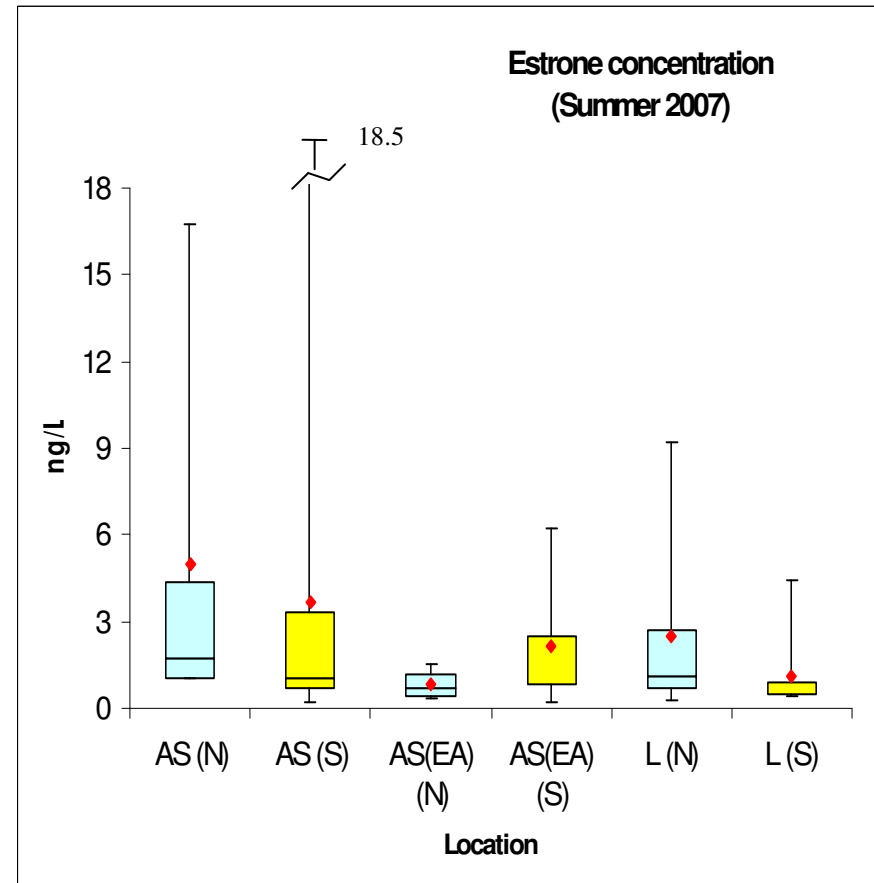


Figure 4.16 Summary of estrone concentrations in Summer 2007 samples.

- Data for FL fraction; AS, activated sludge-based WWTP; AS(EA), treatment based on extended aeration-flocculation; L, lagoon-based WWTP; N, north; S, south; ♦, arithmetic mean; line within data boxes, data median; upper and lower boundaries of boxes, 75th and 25th percentile of data.

Table 4.4 Selected studies highlighting reproductive effects in fish exposed in laboratory to estradiol.

Fish species	Exposure Duration & LOEC (ng/L)	Life stage & Effect	Reference
Medaka (<i>Oryzias latipes</i>)	2 generations; 8.7	P.H. to F1 generation; abnormal sex differentiation	Seki et al., 2005 ³⁵
	1 month; 10	P.H.; all female fish	Nimrod & Benson, 1998 ⁶³
	100 days; 10	P.H.; 10% of males with testis-ova	Metcalfe et al., 2001 ⁶⁴
	20 days; 33.5	P.H.; testis-ova observed in male fish after 14 days	Hirai et al., 2006 ⁶⁵
	21 days; 29.3	R.A.; males with testis-ova	Kang et al., 2002 ⁶⁶
	2 weeks; 817	R.A.; reduced egg production and decreased egg hatch	Shioda & Wakabayashi, 2000 ⁶⁷
	6 days; 15000	E & Y; more phenotypical males than females, intersex gonads	Koger et al., 2000 ⁶⁸
Java-medaka (<i>Oryzias javanicus</i>)	6 months; 16	P.H.; fecundity lower than that of control	Imai et al., 2005 ⁶⁹
Fathead minnow (<i>Pimephales promelas</i>)	19 days; 120	R.A.; inhibition of egg production	Miles-Richardson et al., 1999 ⁷⁰
	3 weeks; 50	A.M.; reproductive failure in competition with unexposed males	Martinovic et al., 2007 ⁷¹
Goldfish (<i>Carassius auratus</i>)	24-28 days; 1000	R.M.; reduced GSI and courting; fewer males with tubercles	Bjerselius et al., 2001 ⁷²
Guppy (<i>Poecilia reticulata</i>)	26-36 days; 850 * 4 weeks; 10000 *	R.A.; no adverse effects on reproduction or young Complete inhibition of male sexual behaviour	Kinnberg et al., 2003 ⁷³
Rainbow trout (<i>Oncorhynchus mykiss</i>)	50 days; 1	Reduced semen volume, sperm density and sperm fertility	Lahnsteiner et al., 2006 ³⁴
Carp (<i>Cyprino cyprino</i>)	3 months; 1000	R.M.; reduced GSI, no milt production	Gimeno et al., 1998b ⁷⁴
	2 months; 9000	J.M.; all juvenile males developed into females	Gimeno et al., 1998a ⁷⁵

- P.H., post-hatch; R.A., reproductive adults; R.M., reproductive males; A.M., adult male; J.M., juvenile male; E, eggs; Y, young; *, single concentration.

Table 4.5 Selected studies highlighting Vtg expression in fish exposed in laboratory to estradiol.

Fish species	Exposure Duration & LOEC (ng/L)	Life stage	Reference
Rainbow trout (<i>Oncorhynchus mykiss</i>)	6 months; 1	parr to F1	Hansen et al., 1998 ²⁹
	21 days; 10	males	Routledge et al., 1998 ⁷⁶
	14 days; 19-26	juvenile	Thorpe et al., 2003 ⁷⁷
Atlantic salmon (<i>Salmo salar</i>)	26 days; 8	parr	Bangsgaard et al., 2006 ⁷⁸
Medaka (<i>Oryzias latipes</i>)	2 generations; 2.9	P.H. to F1	Seki et al., 2005 ³⁵
	21 days; 8.9	males	Seki et al., 2006 ⁷⁹
Fathead minnow (<i>Pimephales promelas</i>)	21 days; 28.6	males	Seki et al., 2006 ⁷⁹
Zebrafish (<i>Danio reio</i>)	21 days; 85.9	males	Seki et al., 2006 ⁷⁹
Eelpout (<i>Zoarces viviparus</i>)	10 days; 50000	males	Korsgaard, 2006 ⁸⁰
Java-medaka (<i>Oryzias javanicus</i>)	6 months; 68	Males P.H.	Imai et al., 2005 ⁶⁹

- P.H., post-hatch.

does not provide conclusive evidence that this is so, i.e. further research is required before Victoria can be assured that hormones in recycled and recyclable water are not a threat to Victorian aquatic environments.

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5. Conclusions

The project has addressed such questions of broad interest to the community and WWTP managers as:

- What is the likelihood that my WWTP effluent is estrogenic?
- Was testing undertaken at the right time (season)? How does this influence the data obtained and the wildlife risk?
- How accurate and precise were the tests?
- Will fish and other organisms be affected by the discharge? How much exposure is required before an effect is observed and is it reversible?

The willingness of consumers to use reclaimed water from municipal wastewater treatment plants is dependent on their confidence that the reclaimed water is safe to use and that the intended applications will be managed to promote sustainable environmental outcomes. Consumer confidence and trust is fragile and likely to only be built over time. So, *what were the drivers for choosing particular chemicals?* The occurrence of endocrine disrupting chemicals (EDCs) in the aquatic environment and their impact on indigenous fauna has generated a significant amount of scientific and public interest since the publication of the book *Our Stolen Future*. Since then, substantial evidence has emerged that many chemicals induce hormone-like effects in wildlife and humans, at the concentrations observed in the environment, i.e. at concentrations much lower than those used in toxicity tests designed to see if the chemicals cause cancer. Chemicals with hormonal activity, i.e. potential endocrine disrupters, include:

- Natural hormones. These can be from any animal, and once released into the environment, chemicals produced by one species can exert hormonal actions

on other animals, e.g. human hormones unintentionally reactivated during the processing of human waste in sewage effluent, may result in changes to fish.

- Natural chemicals, including toxins produced by components of plants (phytoestrogens, such as genistein or coumestrol) and certain fungi.
- Synthetically produced pharmaceuticals that are intended to be highly hormonally active, e.g. components of the contraceptive pill and treatments for hormone-responsive cancers.
- Man-made chemicals and by-products released into the environment.
- The effluent from municipal wastewater treatment plants (WWTPs) is considered the source of much of the EDC input into aquatic environments, and the naturally-occurring, human sex hormones the source of much of the hormonal activity in most WWTP effluents.
- Hence, the choice to focus on the steroid hormones in this project.

What is the likelihood that my WWTP effluent is estrogenic? In short, the likelihood is high, but it also apparent that for the most part levels in Victorian WWTPs are likely to be low (generally less than 10 ng/L EEQ).

Was testing undertaken at the right time (season)? Clearly, the more monitoring that is undertaken, the easier it will be to answer this question. That said, in this study testing was undertaken twice, in Winter and Summer, to assess if there were seasonal effects on water quality.

- The data produced by the Winter testing regime provides information on the hormonal activity of stored waters (generally lagoon systems, and lagoons

associated with many AS plants, particularly in the north of the state). The data produced can, therefore, be used when assessing the risk of using this stored water in the Spring, e.g. as replacement environmental flow or application to land.

- The data produced by the Summer testing provides information on the hormonal activity of waters during peak re-use, and can be used when assessing the risk of recycling this water to land, or into amenity waters (e.g. ponds, urban lakes).

How accurate and precise are the results? This question can also be put another way, how credible are the results? Although implicitly this question questions the credibility of the project team, it is not an unfair question, given the importance of the issue.

- In part the credibility of the results lies in the extensive, international research experience of the lead investigators, particularly the almost two decades of experience measuring persistent organic pollutants (e.g. dioxins, dioxin-like chemicals, DDT etc.) using complex sample preparation and measurement techniques.
- In part, credibility lies within the quality-systems approach taken by Environmental Health & Chemistry's laboratories. The methods used in this project were non-standard (there are as yet no recognised standard methods to do what we were asked to do), and so quality assurance testing was undertaken routinely during the testing program.
- In part, the credibility of our results lies in the long-term collaboration with the National Institute for Environmental Studies (NIES) in Japan, which was used to access some of the tools used in

this investigation, i.e. validation of data by experts in the technique in an international (and internationally recognised) research organisation.

- In part, credibility of technique is provided because other research groups in Australia and New Zealand have also been basing their EDC research on collaboration with the same group at NIES.
- In part, the credibility of our results lies in validation of data trends through a second technique based on different analytical technology i.e. chemical measurement of estrogens and androgens using enzyme-linked immunosorbent assay (ELISA) technology.

That said, we recognise that there are some interesting differences between our results and those found in southern Queensland, and that these may be the result of methodological differences. We recognise that, in part, in future, the credibility of our results (and those of other laboratories) will rest in active, successful participation in inter-laboratory validation trials.

- One conclusion and recommendation from these studies is that national, inter-laboratory validation trials are required. Such trials are, perhaps, best organised by N.A.T.A., funded through the CRC for Water Quality and Treatment, and stimulated by the water authorities themselves.

What is the likely environmental impact of current / future discharge of WWTP effluents into receiving waters? This is a difficult question to address. Although our results suggest that the estrogenic activity of Victorian treatment plants is higher than that observed elsewhere in Australia (although in and of itself this observation may simply reflect the lack of monitoring

undertaken in other states and the difficulties associated with comparing like-for-like WWTPs), they also highlight that the level of hormonal activity, and the concentrations of individual hormones are relatively low.

- All Victorian WWTP effluents studied were hormonally active to some extent, containing both estrogens and androgens.

Typically, in environmental risk assessment one first looks to agreed national or international guideline or trigger values for the type of waters being assessed. In this case, there are as yet no guideline values, although the UK Environment Agency is apparently considering a trigger value of 1 ng/L EEQ in river water (Pers. Comm., Sue Jobling, Brunel University, London). Without guideline values to drive the assessment, then one compares a chemical's concentration in a sample (in this case a WWTP effluent) with data obtained from toxicological experiments in which the concentration known to elicit a specific effect has been determined. In this case, levels of estradiol were typically between the lowest reported level to induce the production of female-only proteins in male fish (plasma vitellogen; 1 ng/L), and the lowest concentration of known to induce intersex in fish (8 ng/L), and consequently there may be some site specific risks, i.e. to a sensitive aquatic receiving environment.

One assumption commonly made when assessing chemicals in WWTP effluents is that the WWTP discharges will be diluted significantly in the receiving environment. Ten-fold (10x) dilution of most of the effluents studied would bring their estradiol concentrations below the lowest reported level to induce plasma vitellogenesis in male fish, and in such cases there may be minimal risk of

endocrine disruption caused by the steroid hormones.

- To assume significant dilution may not be appropriate in some circumstances, e.g. where the discharge represents all, or most of, the environmental flow in a waterway, or where discharges are to enclosed water bodies (e.g. lakes). In such cases, there may be significant risks to aquatic wildlife.

In this study, we did not measure the concentration of one of the most potent steroidal hormones, namely ethinyl estradiol (EE2). This synthetic compound is the active ingredient in many forms of contraceptive pill, and has a potency at least 25 times that of estradiol, and is known to cause physiological impacts at concentrations as low as 0.1 ng/L. Consequently if only relatively small proportion of the observed estrogenicity is due to EE2, the risk of impacts in receiving environments may be greater than might otherwise be anticipated.

- No account is taken of additive or synergistic effects of mixtures of chemicals, i.e. the chemical cocktail found in the effluent may induce effects equal to the sum or, or greater than the effects seen by individual chemicals in the mixture at equivalent concentrations.

No instrument can measure toxicity or other chemical impacts on organisms. Moreover, the *in vitro* assays used in this study are useful as screening tools for monitoring studies, but they are a simplification of the *in vivo* situation, i.e. although they are living systems *in vitro* tests do not have the complexity (in scale or scope) of more complex organisms, and thus do not take into account processes such as bioavailability, metabolism and excretion, or cross-talk between biological pathways, nor address effects that result from multiple mechanisms. Consequently,

to truly assess the risk (hormonal impact) of these WWTP effluents, *in vivo* testing needs to be undertaken, ideally with a representative native species but failing that with a 'standard' species such as the fathead minnow.

The project has provided a large amount of baseline data for Australian researchers and, although undertaken with reasonably limited scope, has identified a number of areas that require further investigation. Based on the data collected in this study, the following areas are worthy of consideration of funding by SmartWater and/or the Victorian Water Trust.

- A study of the hormonal activity of mechanical plants using trickle filter technology (these were not investigated in this study, and are known to have limited removal efficiency for steroid hormones).
- A study of the seasonal variation in hormonal activity at selected WWTPs, e.g. those discharging to freshwater environments, supplying major recycling schemes, or serving major tourist destinations.
- A study exploring the seasonal changes in phytotoxicity of WWTP effluents, and the potential impact on agricultural crops.
- A study of Victoria's riverine environments to ascertain whether there have already been impacts on fish (native and for some exotics (e.g. trout, redfin perch)) in those Victorian waterways currently regularly receiving WWTP discharges (a major knowledge gap identified by this study).
- A study exploring other sources of EDCs in the environment, e.g. from agriculture, forestry, the urban environment.
- A study evaluating fish development when grown in 100% WWTP effluent compared and contrasted to fish developing in natural waters. Although exploring a 'worst-case exposure scenario,' WWTPs have been identified as a reasonably secure water source for aquaculture development in northern Victoria, which in turn could pose an ecological risk if native fish are grown for restocking purposes (through stocking of developmentally impacted fish). DPI has a project plan ready that would cover this knowledge gap 'on the drawing board,' and which would be undertaken by a coalition involving DPI, water authorities and university academics.
- Development of an *in vivo* test system using a representative native fish.
- A study further exploring androgen concentrations in effluent and measurement of impact (cause-effect) using an *in vivo* system.
- A study exploring the behaviour of steroidal hormones in sediments (especially anoxic sediments) in aquatic systems receiving WWTP effluents (to assess the potential for a 'reservoir effect').
- A study of the environmental fate of steroid hormones in field soils, in situ in the presence of co-contaminants (to assess risks to soil health and the potential transport risks from enhanced mobility associated with recycled water).

Ultimately, the study has again highlighted that it is not just the big city that produces hormonally active effluent (as one might intuitively believe), but also smaller communities. Indeed, some of the effluent from WWTPs serving small communities is more hormonally active than that of the larger cities.

- The best advice for treatment plant operators is, “have the hormonal activity of your plant measured.”

When this program began, the ‘watching brief,’ being held in Australia on the topic of endocrine disrupting chemicals and their potential effects on aquatic wildlife was considered too passive by many. It still is, by some. Despite the assurance our results may provide, there is still a need for further extensive on-ground, reassurance research to provide data for higher-level risk assessment by industry and government agencies.

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