



Government of **Western Australia**
Department of **Water**

Development of an 'ecotoxicity toolbox' to characterise water quality for recycling



Looking after all our water needs

Water Science
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Department of Water

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Summary

With water in short supply in many areas of Australia, attention is turning to the use of recycled water for both potable and non-potable uses. Traditional chemical measurements alone are an insufficient basis for environmental risk assessments where trace contaminants are involved, particularly for complex mixtures such as wastewater. Some of the major limitations of chemical measurements are the lack of integration of possible mixture interactions, an inability to account for non-target chemicals (such as metabolites), and detection limits that are sometimes higher than biologically-active concentrations for the most potent chemicals.

In this project, we developed an 'ecotoxicity toolbox' to characterise water quality through the wastewater treatment process. The toolbox assesses a range of toxic responses, including *in vitro* endpoints for cytotoxicity (MicroLumo bioassay), genotoxicity (*umuC* bioassay), photosynthesis inhibition (Max-I-PAM assay) and estrogenic and androgenic endocrine disruption (E-SCREEN and AR-CALUX respectively); as well as *in situ* effects using mosquitofish (*Gambusia holbrooki*) as the sentinel species. Chemical analysis was carried out in parallel for some chemical groups to correlate standard analytical methods with this new approach. Specific wastewater treatment plants (WWTPs) were selected for inclusion in the study to represent a range of treatment processes and discharge water qualities, and potentially different end-uses, including:

- Beenyup WWTP and pilot advanced water treatment plant (AWTP)¹: a major urban wastewater treatment plant, with a planned trial of groundwater replenishment using reverse osmosis (RO) treated wastewater as a potential future drinking source
- Waroona and Harvey WWTPs: rural plants with secondary treatment before environmental discharge to drains
- Gordon Road WWTP: advanced secondary treatment with discharge to groundwater via infiltration ponds, and downstream abstraction for irrigation use.

Samples were taken at various steps in the treatment train to characterise water quality through the treatment process. The field work component to ascertain *in situ* effects using mosquitofish was conducted at those plants with surface water discharge into drains (Harvey and Waroona WWTPs).

The results show:

- There was significant biological activity in raw wastewater, usually higher in the raw wastewater influent to Beenyup AWTP and Gordon Road WWTP compared with Waroona and Harvey WWTPs. There were also significant

¹ Note: for the purpose of this report, Beenyup AWTP and WWTP are hereafter collectively referred to as Beenyup AWTP

concentrations of measured chemical contaminants in raw wastewater at all treatment plants.

- There were differences in removal efficacy between the different treatment plants, with Beenyup AWTP and Gordon Road WWTP usually very effective at reducing the biological activity, with the pond systems at Waroona and Harvey WWTPs less so.
- RO was an effective barrier to biologically-active compounds. None of the chemicals monitored in this study were detected in RO permeate. Genotoxic, phytotoxic, and estrogenic or androgenic activity were likewise not detected in RO water. The low-level cytotoxicity that was detected in two out of four RO permeate samples is not believed to be biologically-relevant.
- The WWTP effluent discharge had no identifiable impact on mosquitofish reproductive morphology at either the Harvey or Waroona WWTP. There was also no observed impact of the WWTPs in terms of the shorter-term biomarker of exposure, vitellogenin content. There was, however, evidence of more diffuse sources of endocrine disrupting compounds (EDCs) entering the stream to which the Harvey WWTP discharges, with male mosquitofish showing low levels of vitellogenin induction both upstream and downstream of the discharge outlet.
- Chemical analysis and *in situ* monitoring were in agreement with the *in vitro* results: chemical analysis further substantiates the removal trends observed by the bioanalytical tools, and *in situ* sampling did not reveal any consistent evidence of endocrine disruption due to discharge of treated wastewater.

This study has demonstrated the usefulness of combining multiple lines of evidence such as chemical analysis, *in vitro* assays, and *in situ* monitoring in the assessment of water quality. Biomarkers of exposure (*in vitro*) and effect (*in vivo*) are complementary, and together provide information with a greater level of ecological relevance than chemical measurements alone. However, it is important that the assays are used with careful consideration of confounding factors, and with a high degree of knowledge of study species and environmental conditions.

The toolbox developed in this project shows promise for application to water recycling initiatives with a range of end-uses and allows a better understanding of the water quality issues involved. Validation and implementation of the toolbox for a variety of case studies is the next step to further verify and promote the utility of this approach.

1 Introduction

With water in short supply in many areas of Australia, attention is turning to the use of recycled water for human consumption and other uses. Water quality monitoring has so far relied mostly on analysis of individual chemicals, but traditional chemical measurements alone are an insufficient basis for environmental risk assessments where trace contaminants are involved, particularly for complex mixtures such as wastewater. The large number of compounds (there are more than 220 regulated chemicals in the *Australian guidelines for water recycling 2008 Phase 2*) and the ultra-low concentrations that can cause biological effects are problematic when relying on chemical analysis alone. But perhaps a more critical limitation of our current approaches in dealing with a complex matrix such as source water is the inability to regulate and detect potential mixture toxicity as well as account for non-target chemicals (such as metabolites and unknowns). There is a clear need to develop additional techniques to overcome these limitations.

Bioanalytical methods are ideal screening tools that can detect the presence of a wide range of contaminants based on their biological effect – irrespective of their chemical identity. Bioanalytical methods have been used in the pharmaceutical industry for decades, and they are now being adapted to water quality assessment. Their main advantage is that they can provide a limited measure of mixture interaction; they can inform the primary effects of biologically-active compounds irrespective of their chemical class and/or interactions; and individual bioassays can potentially provide a sum-measurement of many different compounds. Bioanalytical tools also have limitations, but many of these can be overcome by parallel chemical analysis as well as *in vivo* measurements. In combination with chemical analysis and *in vivo* assessments, ‘unknown’ biologically-active contaminants can be detected and chemically identified, or be taken into account for their *in vitro* biological effects. In addition, bioanalytical tools often have greater sensitivity than *in vivo* methods or even chemical analysis (Figure 1).

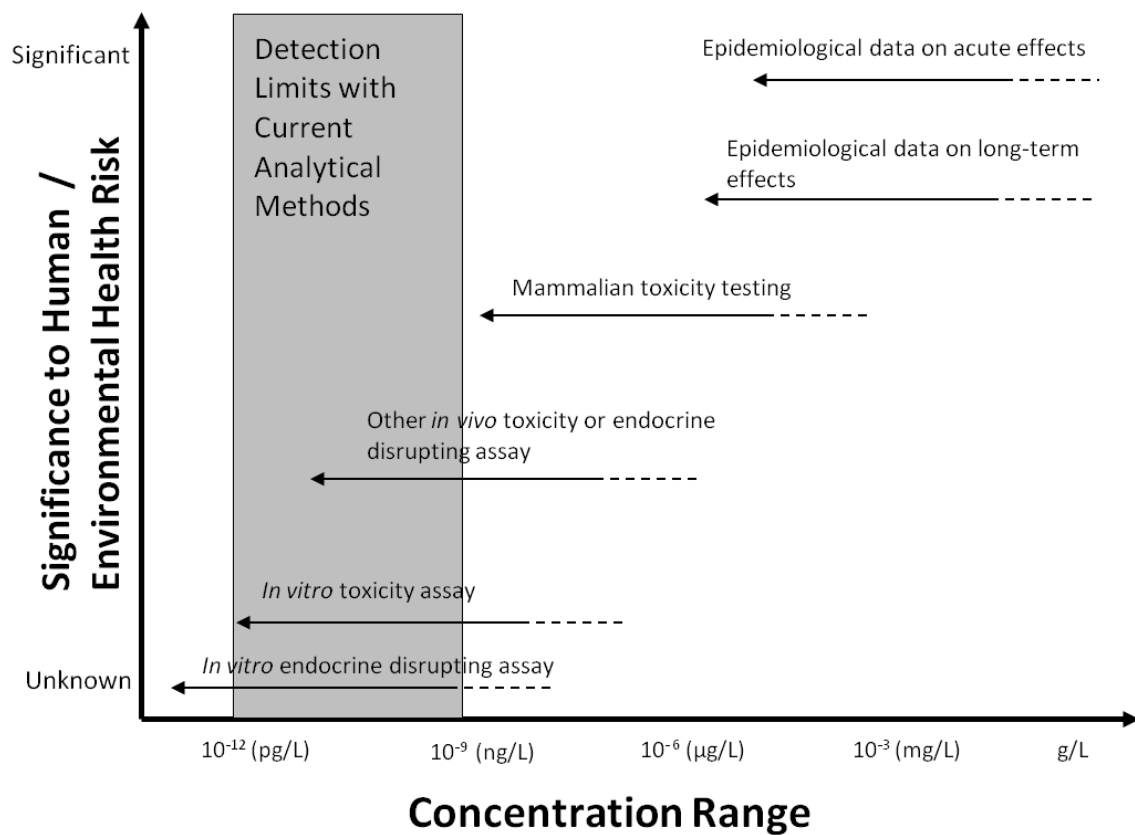


Figure 1: Matrix of significance versus sensitivity of epidemiology, in vivo and in vitro techniques. Based on that from Asano and Cotruvo (2004).

1.1 Background

This project proposes to use an 'ecotoxicity toolbox' to characterise water quality through wastewater treatment processes. The toolbox includes *in vitro* endpoints for basal toxicity (cytotoxicity), genotoxicity, photosynthesis inhibition, and endocrine disrupting effects. Chemical analysis was performed in parallel to correlate standard analytical methods with this new approach. The Beenyup AWTP (which is engaged in the groundwater replenishment trial) and the Harvey, Waroona and Gordon Road WWTPs were monitored to represent a range of treatment methods, discharge water qualities and end-uses in rural and urban settings in Western Australia. Samples were taken at various steps in the treatment train to characterise water quality through the treatment process. For those plants with surface water discharge into drains (Waroona and Harvey), further field work was carried out to assess *in situ* effects using mosquitofish (*Gambusia holbrooki*) as a sentinel species.

This project aims to identify and quantify a wide range of biologically-active contaminants and the effects they might induce on biota during wastewater treatment

and upon discharge (whether it be to an aquifer or surface water), with a particular focus on EDCs. Two of the five *in vitro* bioassays, much of the chemical analysis and the *in situ* fish analysis focused on estrogenic and androgenic endocrine disruptors.

1.1.1 A focus on endocrine disrupting compounds (EDCs)

Anthropogenic compounds in the environment that interfere with the normal function of endocrine systems – EDCs – have the potential to cause ecological harm. Of particular concern are those EDCs that mimic or block the action of endogenous sex hormones (estrogens and androgens), potentially causing reproductive dysfunction in wildlife (Arcand-Hoy & Benson 1998). Native fauna inhabiting aquatic ecosystems are exposed to EDCs and investigative research needs to be undertaken to fully understand the sources of EDCs, their persistence and the risks they pose.

Treated wastewater is recognised as one of the most important sources of EDCs in the environment. Around the world, studies on WWTPs have shown that influent material contains many potent steroid hormones and hormone mimics including natural estrogens (e.g. 17 α -estradiol, 17 β -estradiol, estrone), synthetic estrogen (17 α -ethynylestradiol), androgens (testosterone) and other organic compounds (e.g. the surfactants nonylphenol and octylphenol) (Ying et al. 2002; Sekela et al. 1999; Lee et al. 2004). Chemical and *in vitro* studies worldwide have described the fate and behaviour of EDCs in WWTPs and the efficacy of treatment processes in their removal, demonstrating that measurable concentrations of EDCs are present in WWTP effluent and are discharged to the environment (Pawlowski et al. 2004; Ternes et al. 1999b; Baronti et al. 2000; Sekela et al. 1999), including in Australia (Leusch et al. 2006b; LWA 2007; Tan et al. 2007a; Braga et al. 2005a).

While chemical analysis can provide information on the concentrations of EDCs entering receiving waters, it is important to understand their cumulative effects. A number of *in vitro* techniques have been developed to estimate the total potential effect of complex mixtures of EDCs typical of WWTP effluents (Routledge & Sumpter 1996; Leusch et al. 2006d). *In vivo* studies on potential receptors (e.g. *in situ* studies on fish in the aquatic receiving environment) may be used to understand the actual effects of these compounds. Ideally population and community-level studies are conducted to fully describe the ecological health of a system. Yet these are time-consuming and expensive. As such, certain biomarkers have been described to estimate exposure of organisms to EDCs and the biologically-relevant effects associated with such exposure.

EDCs which act via the estrogen receptor (either by blocking or potentiating responses) are among the most widely studied and induce some of the most pronounced effects in wildlife by impacting directly on reproductive function. Many studies worldwide have used the induction in male fish of a female-specific protein (vitellogenin) as a sensitive biomarker of environmental exposure to estrogenic compounds (e.g. Sumpter & Jobling 1995; Harries et al. 1996; Sole et al. 2001). A number of abiotic and biotic factors may influence the ability of these compounds to exert effects. There is evidence to suggest differences between the potential effects of EDCs across seasons, which may be related to water temperature or reproductive

state of the fish at the time of exposure. Similarly the age, sex and nutritional state of the fish may alter the strength of these effects (Damstra et al. 2002).

One of the most common biomarkers for indicating the biological effects of EDCs is the development of secondary sexual characteristics in male mosquitofish (*Gambusia holbrooki*). Mosquitofish are a widespread invasive pest, inhabiting fresh to brackish waterways across Australia. Male mosquitofish possess an elongated anal fin (gonopodium) which is under androgenic control (Turner 1942; Rosen & Gordon 1953). Development of the gonopodium is delayed or halted under exposure to estrogenic compounds in both laboratory and field studies (Rawson et al. 2008; Doyle & Lim 2002; Batty & Lim 1999; Leusch et al. 2006a).

1.1.2 The 'ecotoxicity toolbox'

Five *in vitro* bioassays were selected for inclusion in the 'ecotoxicity toolbox'. The bioassays were chosen to provide an appropriately wide coverage of biological effects in species relevant to managed aquifer recharge and environmental discharge of treated wastewater (Table 1).

Table 1: Matrix showing the intersection of biological effect versus type of organism for each of the *in vitro* bioassays in the 'ecotoxicity toolbox'

Effect \ Organism	Bacteria	Plants	Vertebrates
Cytotoxicity (acute cellular toxicity)	MicroLumo		
Genotoxicity (damage to DNA)	<i>umuC</i>		
Inhibition of photosynthesis	Max-I-PAM		
Estrogenic endocrine disruption			E-SCREEN
Androgenic endocrine disruption			AR-CALUX

The MicroLumo assay is a measure of non-specific toxicity. It measures inhibition of bioluminescence in the naturally-bioluminescent marine bacteria *Vibrio fischeri*. This inhibition is caused by acute cellular toxicity or interference with energy generation pathways, and is responsive to a wide range of toxic chemicals.

The *umuC* assay is a measure of reactive toxicity (genotoxicity). It measures induction of the SOS response in a genetically modified strain of *Salmonella typhimurium*. The SOS response is a DNA repair mechanism, and is induced in response to single-stranded DNA damage. Although it is a measure of genotoxicity in a bacterial model, the event detected (single-strand DNA damage) represents the potential genotoxicity in all types of cells because the molecular DNA structure is highly conserved. This bioassay therefore spans all three groups in Table 1.

The remaining three bioassays are all measures of specific toxicity: photosynthesis inhibition in the Max-I-PAM, estrogenicity in the E-SCREEN and androgenicity in the AR-CALUX.

The Max-I-PAM assay measures changes in the photosynthetic yield of algae, and is generally responsive to herbicides. Photosynthesis is mostly conducted by plants, but some bacteria are also able to photosynthesise, and the bioassay therefore spans these two groups in Table 1.

The E-SCREEN assay measures proliferation of estrogen-dependent human cells, and is responsive to estrogenic EDCs (e.g. natural estrogens, pharmaceuticals, and industrial estrogen mimics such as nonylphenol and bisphenol A).

Finally the AR-CALUX assay measures induction of an androgenic reporter gene in a genetically-modified human cell line, and is responsive to androgenic EDCs (e.g. natural androgens, pharmaceuticals and other androgen mimics).

1.2 Project drivers

There were two key drivers for this project:

1. Perth water catchments and groundwater have suffered significant water-level reductions as a result of changing rainfall patterns. Reduced rainfall has significantly diminished water availability during the past 30 years in Australia's south-west. At the same time Perth is experiencing an increase in population and competing demands for water. Consequently the Water Corporation is investigating alternative water supplies such as recycled wastewater (including its potential to be used as drinking water after storage in aquifers for a period of time).

2. A large number of rural WWTPs currently discharge treated wastewater to the freshwater environment. The potential for environmental impacts due to the effluent's ecotoxicity is not well understood.

Whether for recycling or discharge to the environment, in both cases an environmental risk assessment is a necessary step in the approvals process. An 'ecotoxicity toolbox' would provide valuable additional information to complement the current chemical analysis approach.

The Beenyup AWTP and Gordon Road, Harvey and Waroona WWTPs were included in this project. They were selected to enable consideration of a range of wastewater treatment technologies and discharge types, for maximum transferability to other WWTPs, and for their relevance to Water Corporation environmental and recycling initiatives. In summary, Beenyup AWTP was selected due to the potential recycling of RO-treated wastewater for groundwater replenishment. Gordon Road WWTP was selected because wastewater is infiltrated at this site and later abstracted by third parties for irrigation use. Harvey and Waroona WWTPs discharge to freshwater streams.

1.3 Project aims

The project's purpose was to measure any change in biologically-active contaminants and particularly EDCs through the wastewater treatment process. This was achieved through the following:

1. Measure biological activity in raw wastewater and in waters treated to various qualities for water recycling

Biological activity was measured by *in vitro* bioassays in raw wastewater, and at different stages during the treatment process through to final effluent at the AWTP and the three WWTPs.

2. Evaluate the use of an 'ecotoxicity toolbox' to assess the potential for environmental impacts from treated wastewater in the freshwater environment

This component incorporated the suite of *in vitro* bioassays into an 'ecotoxicity toolbox'. The development of this toolbox will provide the capacity to assess the potential for environmental impacts resulting from wastewater recycling and discharge. The toolbox consists of *in vitro* tests for cytotoxicity, genotoxicity, phytotoxicity and both estrogenic (feminising) and androgenic (masculinising) endocrine disruption.

Targeted chemical analysis using high performance liquid chromatography-multidimensional mass spectrometry (HPLC-MS/MS) techniques were evaluated and compared with the ecotoxicity tests where possible. This enabled the data generated from the ecotoxicity toolbox to be compared with chemical measurements. It also provided an opportunity to identify the biologically-active chemicals.

3. Compare the results from chemical analysis and in vitro bioassays with biomarkers in fish collected in the receiving environment

In vivo biomarkers of exposure and effect were measured in aquatic systems receiving treated WWTP effluent. Specifically, the following endpoints were measured in mosquitofish collected in the receiving environment: vitellogenin content (a biomarker of exposure to estrogenic EDCs) in male mosquitofish, a set of *in situ* biomarkers of effect (development of secondary sexual characteristics), and the demography of populations of mosquitofish (sex ratios, male developmental stage). These endpoints provided a measure of the presence and effects of EDCs in waters receiving effluent from the Harvey and Waroona WWTPs south of Perth, which were compared with the results from both chemical analysis and *in vitro* bioassays.

2 Materials and methods

2.1 Sampling for *in vitro* and chemical analysis

2.1.1 Sampling sites

Selection of the treatment plants considered the range of treatment technologies common in south-west Western Australia, for maximum transferability to other similar plants. The other major factor in their selection was variability in the type of end-use/receiving environment, covering all the major current or planned discharge types in the region. The variability in treatment technology strongly influences treated water quality, but clearly the variability in plant capacity and catchment characteristics also affects influent water quality. The treatment plants represent the extent of variability in catchments – from small rural communities to major urban areas. Those selected and their major treatment and discharge characteristics are shown in Table 2.

Table 2: Characteristics of the treatment plants monitored in this project

Treatment plant	Treatment	Discharge
Beenyup AWTP (metropolitan)	Activated sludge WWTP. Raw, primary, secondary, microfiltration and RO (pilot plant).	Ocean. In future, injection recharge of RO water to the confined Leederville Aquifer for potable use.
Gordon Road WWTP (urban and rural)	Activated sludge, oxidation ditch.	Superficial Aquifer infiltration, with abstraction for irrigation.
Waroona WWTP (rural)	Pond-based, with alum dosing.	Freshwater stream
Harvey WWTP (rural)	Pond-based, alum dosing and chlorination.	Freshwater stream

The in-plant sampling locations are summarised in Table 3.

Table 3: Sample locations

	Beenyup	Waroona	Harvey	Gordon Road
Raw	X (raw feed)	X (at entry into pond 1)	X (at entry into pond 1)	X (raw feed)
Partially treated	X (primary)	X (pond 2 discharge)	X (pond 2 discharge)	X (oxidation ditch)
Effluent	X (clarifier discharge)	X (pond 3 discharge)	X (pond 4 discharge)	X (clarifier discharge)
Tertiary treated (RO)	X			
Environment		X u/s drain * X d/s drain *	X u/s drain * X d/s drain *	

Notes: d/s = downstream (taken at mixing point of effluent with native water); u/s = upstream. * = also site for *in situ* mosquitofish sampling (a reference site, i.e. Serpentine National Park, was also included for *in situ* mosquitofish sampling).

Through the treatment process samples were taken once in each season over a year at each location in quadruplicate: two natural samples and two samples spiked with deuterated hormone surrogates. Twenty-four-hour composite samples were taken at Beenyup AWTP; grab samples were taken at Waroona, Harvey and Gordon Road WWTPs.

2.1.1.1 Beenyup advanced wastewater treatment plant (AWTP)

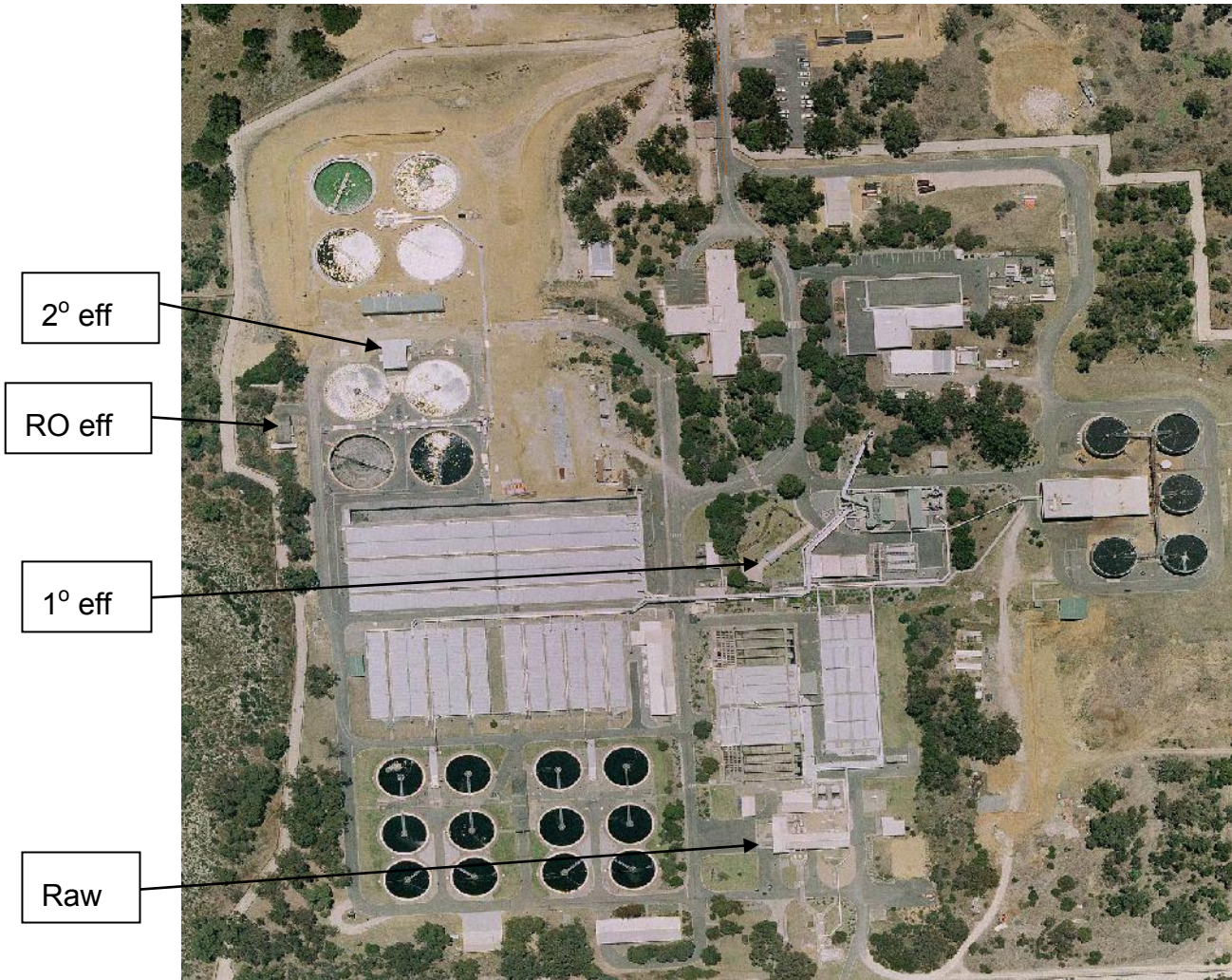


Figure 2: Annotated aerial photograph of Beenyup AWTP

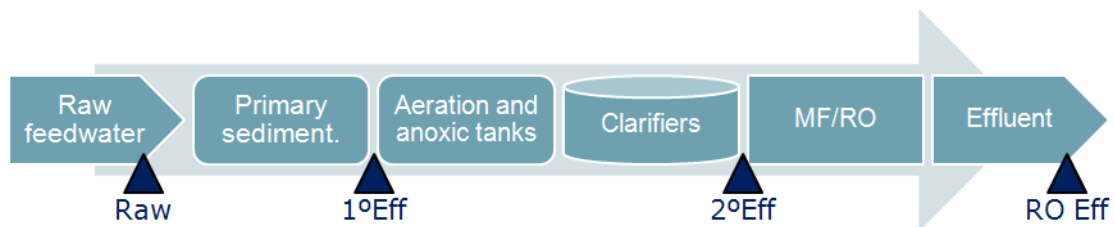


Figure 3: Simple schematic of treatment at Beenyup AWTP with sampling locations

RO-treated wastewater from the Beenyup AWTP is being used to trial groundwater replenishment, or managed aquifer recharge. The wastewater is treated by

microfiltration, RO and potentially advanced oxidation, and then injected into the Leederville Aquifer at a location remote from existing drinking water bores, where the water will be further treated by natural groundwater processes before extraction.

This project aims to monitor water quality throughout the treatment train before the water is injected into the aquifer, to help build an understanding of the effectiveness of each stage of the treatment process.

The project complements the Premier's Collaborative Research Program project 'Characterising treated wastewater for drinking purposes following RO treatment'. The project also has synergies with a new Water Quality Research Australia project which has also received Raising National Water Standards program funding and focuses on human health and the development of new assays <www.chemicalsinrecycledwater.info>.

The Beenyup AWTP is located on Ocean Reef Road in Craigie, approximately 20 km north of Perth's CBD. It serves Perth's rapidly developing northern suburbs from Quinns Beach through to Scarborough and inland through Dianella and Bayswater to the foothills east of Midland. Wastewater is predominantly sourced from households. Most non-residential wastewater is sourced from the food manufacturing/processing and retail (restaurant) industries.

The Beenyup AWTP is an advanced secondary treatment plant. Capacity is currently 120 ML/day (serving a population of about 600 000) with a planned upgrade to treat 200 ML/day. The plant process involves screening, grit removal, activated sludge treatment with aerated and anoxic zones and clarification. Like other treatment plants across the state, the Beenyup AWTP is subject to regulation and licensing by the Department of Environment and Conservation (DEC).

During the project, a small pilot RO plant treating about 100 kL/day was set up to treat wastewater from the Beenyup AWTP's discharge line. The treatment process consisted of chloramination, pH control (sulfuric acid dosing), microfiltration (Memcor hollow fibre PVDF membranes), antiscalant dosing and RO (two stage, Hydranautics four-inch spiral wound composite polyamide membranes, ~80% recovery, 19.7 L/m²/hr average flux). This pilot plant was established to generate water for research purposes only and provided the RO samples for this project.

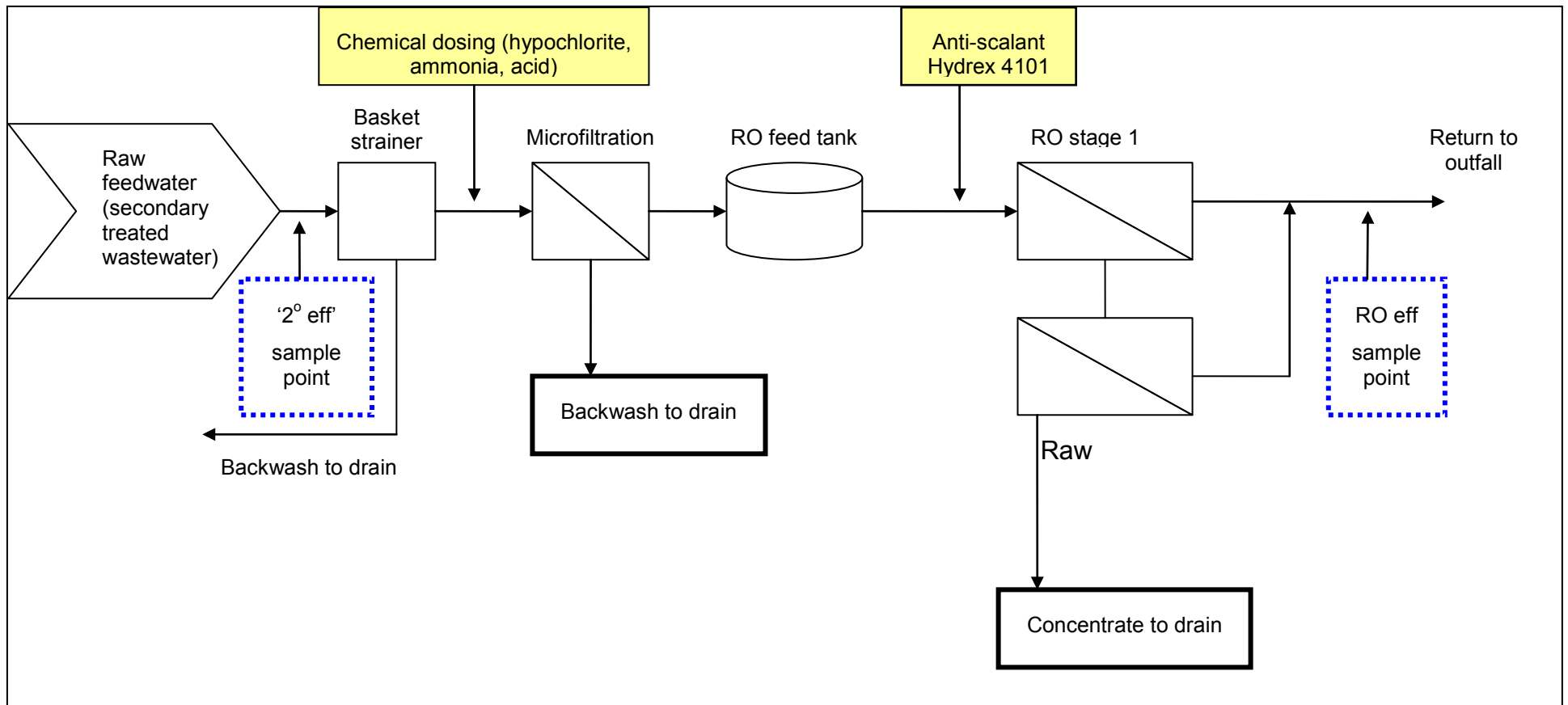


Figure 4: The advanced treatment process at Beenyup AWTP, with dashed boxes indicating sampling locations

2.1.1.2 Waroona wastewater treatment plant (WWTP1)

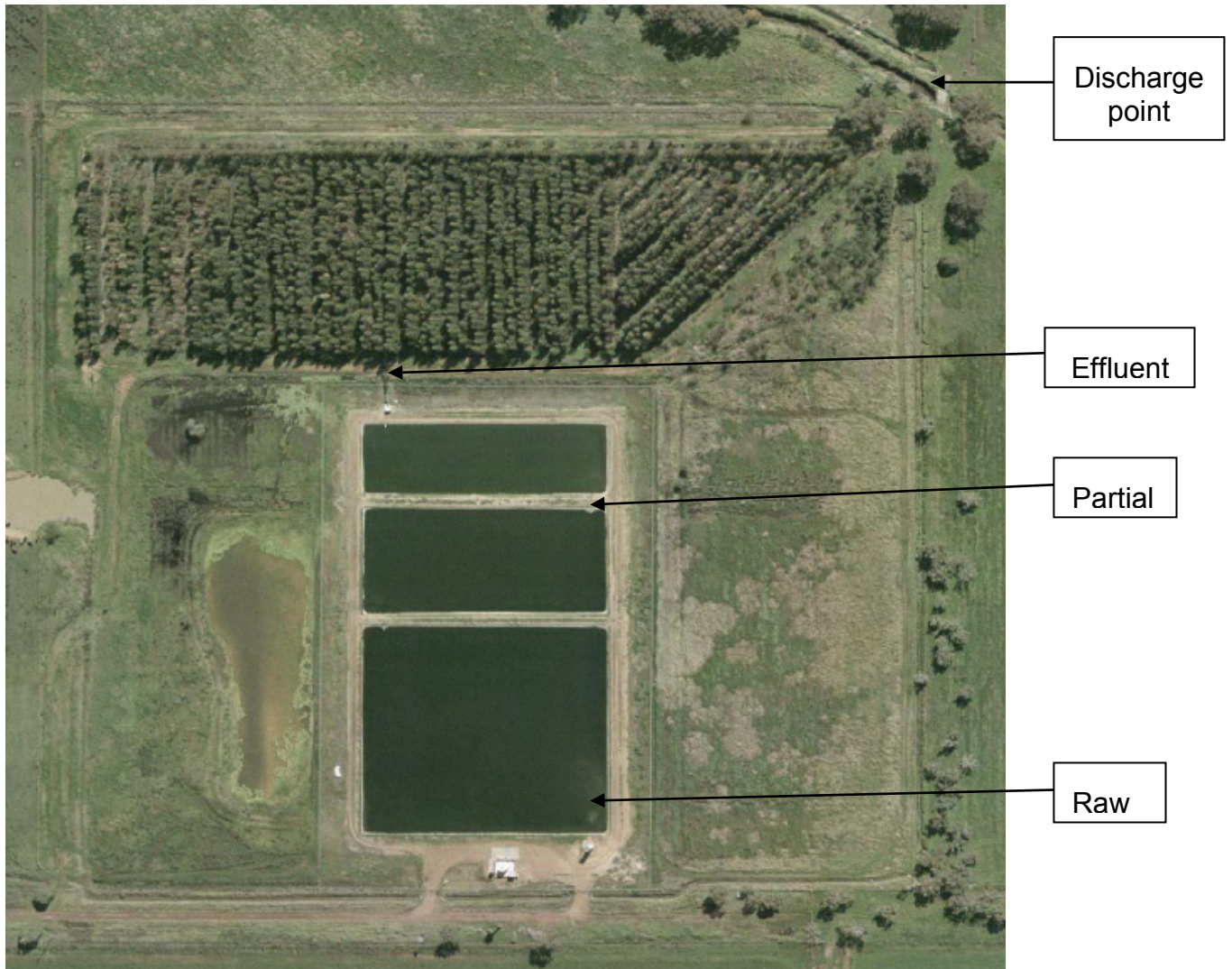


Figure 5: Annotated aerial photograph of Waroona WWTP

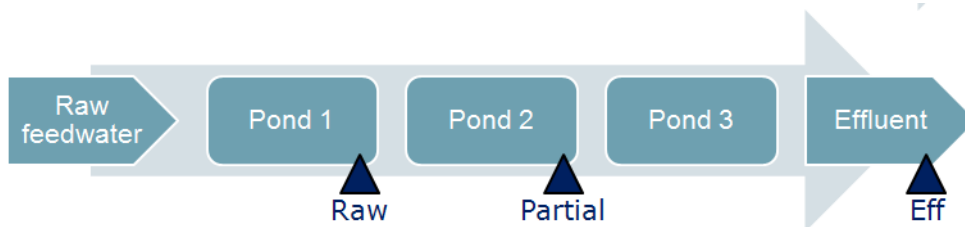


Figure 6: Simple schematic of treatment at Waroona WWTP (WWTP 1) with sampling locations

The Waroona WWTP is designed to service a nominal population of 5000. At 30 June 2007, there were 461 services connected. The average daily inflow to the plant for the period July 2006 to June 2007 was 227 kL.

The Waroona WWTP treats wastewater to a secondary standard and consists of three treatment ponds in series with alum dosing. Treated wastewater is discharged to an agricultural drain after passing through a tree-lot disposal area.

Waroona WWTP uses a combination of aerobic and anaerobic processes involving a broad range of microorganisms. These microorganisms and processes are basically the same as those responsible for the self-purification of rivers and lakes polluted by urban wastewaters. However, wastewater treatment technologies are designed to create the best-possible conditions for microbial growth and thus optimise the treatment processes. This leads to the removal of organic carbon, nutrients and pathogenic microorganisms and thus the production of an effluent suitable for discharge to the environment.

Ponds are less costly to construct and easier to operate than many other treatment systems. Oxidation ponds use algae and wind action to introduce oxygen to the pond liquid. The wind and inlet flows create currents within the pond that help to mix the wastewater over the full area. The quality of effluent from an oxidation pond depends on the extent of mixing and how long the wastewater has been held in the pond.

Faecal coliform bacteria, indicators of the possible presence of pathogens, die off naturally with time and sunlight. As such, the longer the wastewater stays in the pond, the more microbes are killed. Dissolved nutrients in the wastewater such as nitrogen and phosphorus feed green algae (microscopic plants floating and living in the water). Like other plants they release oxygen as a waste product, which is used by helpful bacteria to break down the incoming waste.

2.1.1.3 Harvey wastewater treatment plant (WWTP2)



Figure 7: Annotated aerial photograph of Harvey WWTP

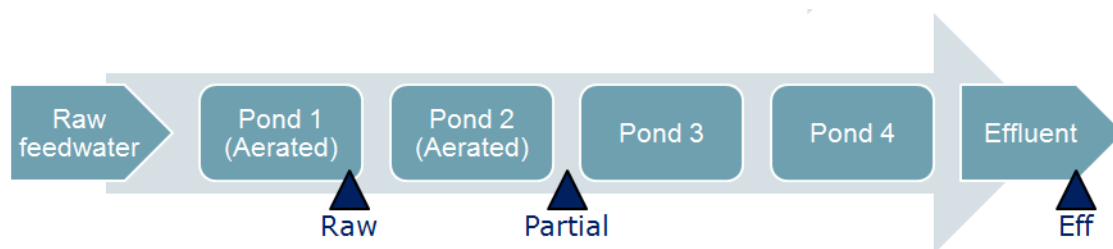


Figure 8: Simple schematic of treatment at Harvey WWTP (WWTP2) with sampling locations

The Harvey WWTP is designed to service a nominal population of 5500. At 30 June 2007, there were 1016 services connected. The average daily inflow to the plant for the period July 2006 to June 2007 was 621 m³.

The Harvey WWTP treats wastewater to a secondary standard and consists of four treatment ponds that the wastewater passes through sequentially. Treated wastewater is discharged into the Harvey diversion drain.

2.1.1.4 Gordon Road wastewater treatment plant (WWTP3)

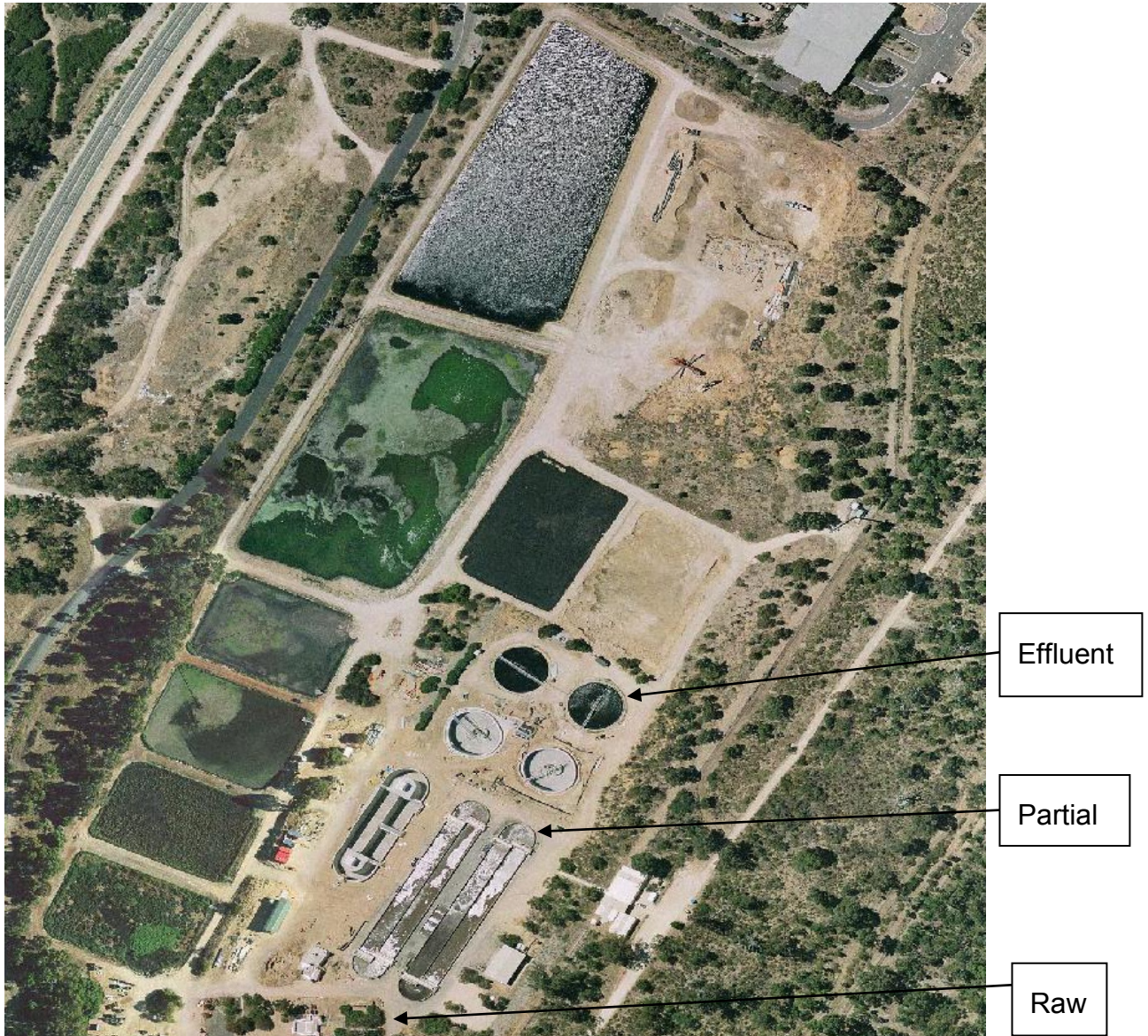


Figure 9: Annotated aerial photograph of Gordon Road WWTP

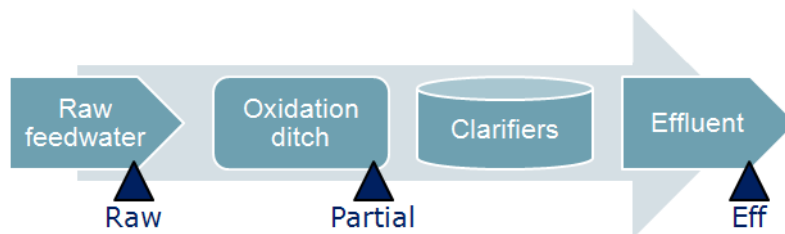


Figure 10: Simple schematic of treatment at Gordon Road WWTP (WWTP3) with sampling locations

The Gordon Road WWTP is located at Mandurah, a regional city 74 km south of Perth. DEC licenses the Water Corporation to operate this WWTP (licence 5950/9) under provisions of the *Environmental Protection Act 1986* (WA).

The Gordon Road WWTP is designed to service a nominal population of 40 000. At 30 June 2007, there were 17 367 services connected. The average daily inflow to the plant for the reporting period was 6513 kL.

The plant treats wastewater to an advanced secondary quality and consists of two oxidation ditches and two clarifiers. Treated wastewater is infiltrated through a series of infiltration lagoons.

Currently Gordon Road WWTP receives inflow of between 7 to 8.5 ML/day. Wastewater is treated to a secondary level of treatment using an activated sludge process and the treated wastewater is discharged to infiltration ponds. Oxidation ditches are used to remove nutrients and organic matter from the wastewater. An oxidation ditch is a variant of the extended aeration type of wastewater treatment plant that uses long sludge-retention times to break down biodegradable material in wastewater. The ditch may be fully aerated or have aerobic and anoxic zones to facilitate nitrification and denitrification processes.

2.1.2 Water sampling

Sample collection and extraction was performed by Department of Water staff. Samples were collected over a 12-month period in five sampling events, covering autumn (twice), winter, spring and summer (Table 4). It is important to note that this study focuses on the aqueous phase only, because solids are mostly removed by the subsequent filtration steps required during concentration, using solid phase extraction (SPE) cartridges.

Table 4: Sampling dates for each site for the *in vitro* bioassays and chemical analysis

	Beenyup	Waroona	Harvey	Gordon Road	Environment
Mar 08	YES	no	no	no	no
Jun 08	YES	YES	YES	YES	no
Sep 08	YES	YES	YES	YES	no
Nov 08 – Jan 09	YES	YES	YES	YES	YES
Mar – Apr 09	no	YES	YES	YES	no

Several stages of the treatment trains were sampled at each plant (Figure 3, Figure 6, Figure 8, Figure 10 and Table 3), including raw, partially- and fully-treated wastewater, and after RO at the AWTP. Water samples (1 L for raw wastewater, 2 L for all other samples) were collected in methanol-rinsed glass bottles by a flow-averaged autosampler (Beenyup AWTP) or by grab samples (all others) and immediately processed. Samples were filtered and extracted by solid-phase extraction using 20cc Oasis HLB SPE cartridges (Leusch et al. 2006d). The SPE cartridges were then sent to Entox in Queensland and UNSW for elution.

2.1.3 Sample elution

The samples were eluted in Queensland and UNSW (March 2009 sampling only) and reduced to a final volume of 500 μL . The elution process and solvents were altered over the project to optimise the process for both bioassay and chemical analysis. The first two sample sets (March and June 2008) were eluted with methanol, acetone and hexane, blown dry and made up to a final volume of 500 μL of ethanol. For these sampling periods ethanol was used as the solvent blank.

The remainder of the sample sets were eluted using methanol only and reduced to a final volume of 500 μL of methanol. The solvent blanks for these samples were 10 mL of methanol reduced to a final volume of 500 μL .

2.1.4 Fractionation experiment (December 2008 samples only)

As the elution procedure changed in December 2008 (to methanol only, see above), a fractionation experiment was conducted to determine if compounds of interest were not recovered by changing the elution process. A second fraction was collected from the Beenyup AWTP and Waroona WWTP September 2008 samples. After the methanol fraction was collected, 2 x 5 mL acetone:hexane fractions were collected separately. This fraction and the solvent blanks were reduced to 500 μL and treated as another sample in the bioassays. These samples showed very low to no activity and it was concluded that the methanol-only extraction method was satisfactory for optimal extraction of the compounds of interest (data not shown).

2.2 Chemical analysis

Two separate chemical analyses were performed on all samples: one screen for androgenic and estrogenic hormones conducted at UNSW, and another for herbicides conducted at Queensland Health Forensic and Scientific Services (QHFSS).

2.2.1 Analysis of androgenic and estrogenic hormones

A range of androgenic and estrogenic hormones were analysed by liquid chromatography-mass spectrometry-mass spectrometry (LC-MS/MS). LC-MS/MS involves chromatographic separation of the mixture components before ionisation in the mass spectrometer. Mass spectrometry is a very specific technique for the detection and identification of organic compounds. It provides not only molecular weight information but also a wealth of structural detail that together give a unique fingerprint for each analyte. It is an established technique for the analysis of complex mixtures, holding a prime position in analytical chemistry because of its combination of sensitivity, wide range of applicability and versatility. This is particularly so in the areas of organic, environmental and biochemistry.

The analytical method adapted for this project was based on a method developed by the Southern Nevada Water Authority. However the published method included only four estrogenic hormones (estradiol, estriol, estrone and ethinylestradiol) as well as

testosterone. Accordingly, the additional analytes to be monitored for this project (as listed below) were added by further method development undertaken at UNSW.

The full list of estrogenic and androgenic hormones analysed (with the limit of reporting) were: the androgens androstenedione (1 ng/L), androsterone (50 ng/L), etiocholanone (100 ng/L), testosterone (1 ng/L), dihydrotestosterone (50 ng/L) and testosterone propionate (5 ng/L); the pharmaceuticals trenbolone (5 ng/L) and 17 α -ethynylestradiol (5 ng/L); and the estrogens 17 β -estradiol (5 ng/L), 17 α -estradiol (5 ng/L), estrone (5 ng/L) and estriol (50 ng/L).

Quantitative analysis was carried out using an isotopic dilution technique to account for extraction losses and any LC-MS/MS matrix effects. The LC-MS/MS instrument consisted of an Agilent LC series 1200 system coupled with an Applied Biosystems API 4000 Q-Trap mass spectrometer. The LC column used in the analysis was a Luna C18, 150 mm x 4.6 mm, 5 μ m, 100A column (Biolab) with security guard cartridges C18 4 mm x 3 mm (Biolab). Mobile phases were methanol and Milli-Q[®] water; both containing 0.1% formic acid. Atmospheric pressure chemical ionisation (APCI) was used as the ionisation source in the mass spectrometer. Quantification was carried out using the Applied Biosystems Analyst 1.5 software. A typical chromatogram showing all of the compounds determined is shown in Figure 11.

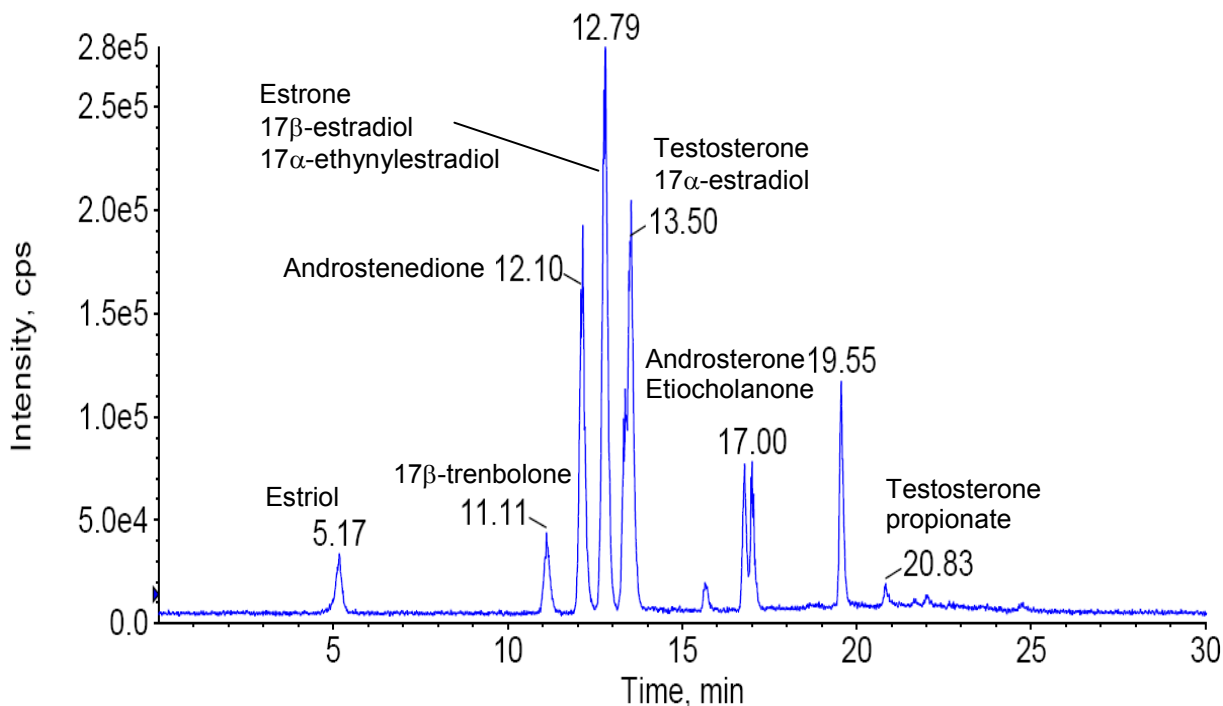
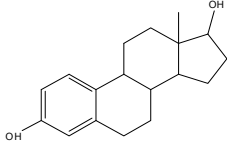
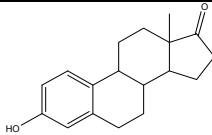
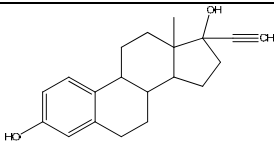
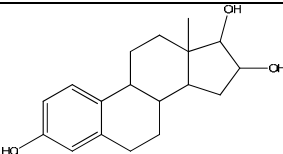
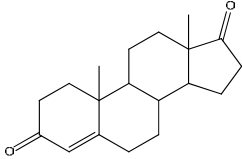
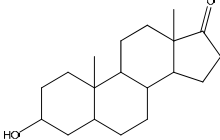
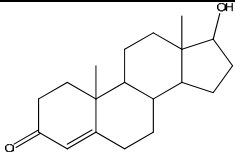
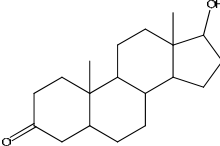
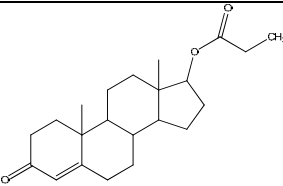
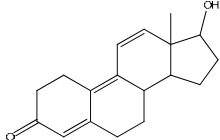


Figure 11: Chromatogram showing retention times and intensities of steroid estrogens and androgens

The key analyte-specific parameters are presented in Appendix A. For most analytes, two transitions were monitored to ensure a very high degree of certainty in correct chemical species identification. Deuterium (D)-labelled internal standards were added and used for isotope-dilution quantification.

Table 5: Name, chemical abstract service registry number (CASRN) and two-dimensional structure of androgenic and estrogenic hormones measured in this project

Chemical name	CASRN	2-d structure
17 α -/17 β -estradiol	57-81-0 / 50-28-2	
Estrone	53-16-7	
17 α -ethynylestradiol	57-63-6	
Estriol	50-27-1	
Androstenedione	63-05-8	
Androsterone / Etiocholanolone	53-41-8 / 53-42-9	
Testosterone	58-22-0	
Dihydrotestosterone	521-18-6	
Testosterone propionate	57-85-2	
Trenbolone	10161-33-8	

2.2.2 Herbicide analysis

The following pesticides were measured: flumeturon, diuron, simazine, atrazine, desethyl atrazine, desisopropyl atrazine, hexazinone, tebuthiuron, ametryn, prometryn, bromacil, terbutryn, metolachlor and imidacloprid. The reporting limit for all compounds was 25 ng/L.

The compounds were determined by HPLC-MS/MS using an AB/Sciex API300 mass spectrometer (AB/Sciex Concord, Ontario, Canada) equipped with an electrospray (TurboSpray) interface coupled to a Shimadzu LC10AD HPLC system (Shimadzu Corp., Kyoto, Japan).

Separation was achieved using a 5 μ m 150 \times 3 mm Aquastar column (Thermo Scientific, Australia) run at 40°C, and a flow rate of 0.6 mL/minute with a linear gradient starting at 10% B for 1.0 minutes, ramped to 100% B in 6 minutes, held for 2.5 minutes and then to 10% B in 0.2 minutes and equilibrated for 4 minutes. (A = 10% methanol /HPLC grade water, B = 90% methanol/ HPLC grade water, both containing 5 mM ammonium acetate.)

The mass spectrometer was operated in the positive ion, multiple reaction-monitoring mode using nitrogen as the collision gas with conditions as listed in Appendix B. Positive samples were confirmed by retention time and by comparing transition intensity ratios between the sample and an appropriate concentration standard from the same run. Samples were reported as positive if the two transitions were present, retention time was within 0.15 minutes of the standard and the relative intensity of the confirmation transition was within 20% of the expected value. The value reported was that for the quantification transition.

2.3 Bioanalytical tools

Samples were tested using five *in vitro* bioassays: the MicroLumo for bacterial cytotoxicity, the *umuC* assay for genotoxicity, the Max-I-PAM for inhibition of photosynthesis, the E-SCREEN for estrogenicity and the AR-CALUX for androgenicity.

2.3.1 MicroLumo

The MicroLumo assay is a modification of the cuvette-based Microtox assay ISO 11348-3:1998. In brief, *Vibrio fischeri* bioluminescent bacteria (JW Industrial) were exposed to sample extracts in a 96-well plate. After a 15-minute incubation, luminescence was measured in each well. The amount of luminescence is directly proportional to the number of surviving bacteria. The modified assay has previously been applied to wastewater extracts (Escher et al. 2008b).

For quality assurance and quality control (QA/QC), samples were tested at six concentrations in duplicate within each assay, and analysed in two independent assays. The positive control compound was phenol, with variation of EC50 within acceptable limits for all tests (EC50 = 72.8 \pm 26.7 mg/L phenol). All solvent blanks were below the detection limit (10% inhibition of luminescence) at the highest sample application volume of 2.8% of total final test volume.

2.3.2 *umuC* assay

The *umuC* assay method is adapted from the method described by ISO 13829:2000. The recombinant bacteria *Salmonella typhimurium* TA1535/pSK1002 is used where the plasmid, pSK1002, contains an *umuC*-lacZ fused gene. Any transcription of *umuC* will also cause production of β -galactosidase, which can be measured using an integrated reporter system associated with the *umuC* gene. The assay was run both with and without inclusion of liver 9000 \times g supernatant fraction (S9), prepared from rats treated with Aroclor 1254 (Moltox Inc. USA). S9 metabolises the samples; therefore the results from the inclusion of S9 are possibly more relevant from a vertebrate health perspective, if it is assumed the water will be ingested.

For QA/QC, samples and blanks were tested at six concentrations in duplicate within each assay, and analysed in two independent assays. The positive control compounds were 2-aminoanthracene (2AA, used in the +S9 incubation; 0.2 ng/L) and 4-nitroquinoline 1-oxide (4NQO, used in the -S9 incubation; 0.05 ng/L). The plate is considered valid when the induction ration of the positive control is >2 . All solvent blanks were below the detection limit at the highest solvent volume (1.9%), and all values reported were from valid plates (positive control induction >2).

2.3.3 Max-I-PAM

A fluorescence-based photosynthetic yield analysis technique was applied for the sample phytotoxicity assessment using the Maxi-Imaging-PAM chlorophyll fluorometer (Max-I-PAM) (Schreiber et al. 2007). The Max-I-PAM bioassay allows sensitive assessment of the phytotoxicity of PSII-impacting herbicides, which bind to the quinine binding site of PSII in photosynthesis and inhibit energy conversion. The assay has previously been applied for detection of herbicidal pollutants in aquatic environments (Escher et al. 2006; Muller et al. 2007).

The positive control compound was the herbicide diuron (EC₅₀ 16.78 \pm 1.42 μ g/L), with the freshwater chlorophyte *Chlorella vulgaris* as the test species. The regulatory levels for diuron are 2.0 μ g/L residue for water use in irrigation and 0.2 μ g/L (low reliability) for freshwater ecosystems in the ANZECC/ARMCANZ guidelines (2000). The *Australian guidelines for water recycling 2008 Phase 2* recommends a human health guideline of 30 μ g/L for diuron.

For QA/QC, samples and solvent controls were tested in eight dilutions in duplicate within each assay, and analysed in two independent assays. All solvent blanks were below the detection limit (1.4 μ g/L diuron) and the variation of the EC₂₀ (quantification level) of the positive control (diuron) was within acceptable limits (two standard deviations).

2.3.4 E-SCREEN

The E-SCREEN assay used the method described by Leusch et al. (2006d) adapted from Korner et al. (1999) and Soto et al. (1995). A human breast cancer cell line MCF7BOS was used to detect estrogenic compounds. This cell line only proliferates in the presence of estrogenic stimulation. Cell proliferation was measured after six days of incubation with the sample using a standard cell viability assay (MTS).

The positive control compound in the E-SCREEN was 17 β -estradiol (EC₅₀ 6.54 \pm 0.5 ng/L). The *Australian guidelines for water recycling 2008 Phase 2* recommends a human health guideline of 175 ng/L for 17 β -estradiol.

For QA/QC, samples were tested at 10 concentrations in triplicates within each assay, and analysed in two independent assays. Positive control 17 β -estradiol (E2) and negative control ethanol were also tested in each assay run. All solvent blanks were below the detection limit (1 ng/L) and only data from plates compliant with Shewhart control charts were used. If the plate's QC values (EC₅₀, minimum absorbance, maximum absorbance, and fold induction) deviated by more than two standard deviations from the assay average, the data was rejected and the assay was repeated.

2.3.5 AR-CALUX

The AR-CALUX assay was based on the standard CALUX protocol (Sonneveld et al. 2005). The AR-CALUX bioassay is based on a U2OS cell line that has been genetically modified with a luciferase reporter gene linked to an androgen-responsive element. When exposed to androgens (or androgen mimics), the reporter gene is activated and the cells produce luciferase. The cells were exposed to the sample for 24 hours and the amount of luciferase at the end of the incubation period was measured in a luminometer.

The positive control in the AR-CALUX was dihydrotestosterone (DHT; EC₅₀ of 47.7 \pm 3.1 ng/L). There is no guideline for DHT, but the *Australian guidelines for water recycling 2008 Phase 2* recommends a human health guideline of 7 μ g/L for testosterone, which in the AR-CALUX is equivalent to 1500 ng/L DHT equivalents.

For QA/QC, samples were tested at five concentrations in duplicate within each assay. Positive control DHT and negative control ethanol were also tested on each assay plate. All solvent blanks were below the detection limit (2.5 ng/L) and only data from plates compliant with Shewhart control charts were used. If the plate QC values were outside of the acceptable range for this assay (EC₅₀ within two standard deviations of the assay average, induction at least six-fold, and r^2 of the standard curve at least 0.98), the data was rejected and the assay was repeated.

2.4 *In situ* sampling and analysis

2.4.1 Fish sampling

The Harvey and Waroona WWTPs, which are surrounded by agricultural land south of the Perth metropolitan area, were selected for *in situ* investigation (Figure 12). Both WWTPs discharge treated wastewater to highly modified streams and initial investigations indicated the presence of mosquitofish populations in reaches upstream and downstream of the discharge points. Mosquitofish were sampled over a range of up to 150 m immediately downstream of each WWTP's discharge point (figures 13 and 14). The upstream site for each WWTP was selected for sampling based on the accessibility and presence of mosquitofish, with considerations of low

potential of population mixing between the two sites on the same stream (figures 13 and 14). In addition, a reference site was selected on the Serpentine River adjacent to the Serpentine National Park's entrance (figures 12 and 15). The influent water from the site originates from the national park, with no influence of treated wastewater or agricultural activities. Mosquitofish were, therefore, collected from a total of five sites.

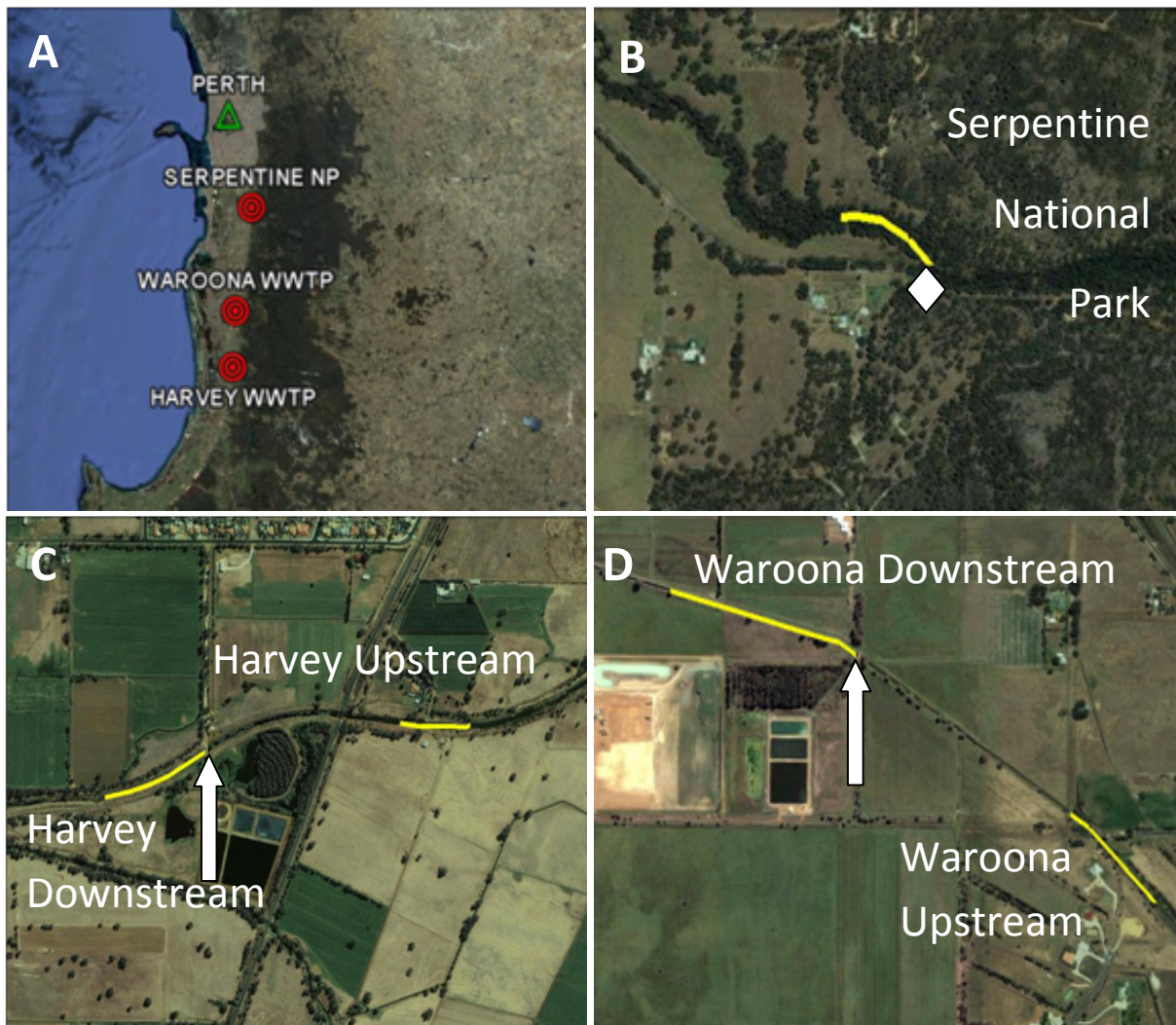


Figure 12: Location of the mosquitofish sampling sites

Notes: A. Location of the sampling sites used in the study; B. Location of the reference site on the Serpentine River (white marker denotes the entry to the Serpentine National Park); C. Location of upstream and downstream sites at the Harvey WWTP (arrow denotes position of effluent discharge point); D. Location of upstream and downstream sites at the Waroona WWTP (arrow denotes position of effluent discharge point).

Mosquitofish were collected at each sampling site on three sampling occasions: September 2008, March 2009 and July 2009. Sampling was conducted on an additional occasion (December 2008), however parts of these samples were lost due

to equipment failure of the cryo-freezer. Only the physical and chemical data for this sampling period are therefore reported. Physical and chemical parameters (temperature, dissolved oxygen, conductivity and pH) were measured at each site before fish sampling. On each sampling occasion, catch effort continued until at least 100 individuals were collected or until the catch per unit effort became too low to justify further sampling. Where catch rates were very low, a second sampling effort was attempted within one week of the original sampling time. Catch was made with a combination of aquatic dip nets and yabby traps baited with tinned tuna. All mosquitofish captured were kept and all other species (fish and invertebrates) returned to the waterway. Fish were initially sorted in the field with 20 adult males (identified by the presence of an elongated anal fin) and six females (identified by a black spot on the ventral side of the abdomen) were placed in individually labelled cryovials and snap-frozen in liquid nitrogen. The remainder were placed in plastic bags on ice for transport to the laboratory where they were immediately transferred to a -80°C freezer.



Figure 13: Sampling locations at Harvey WWTP

Notes: A. Upstream of WWTP; B. Harvey WWTP discharge point; C. Downstream of Harvey WWTP (view is upstream toward discharge point).

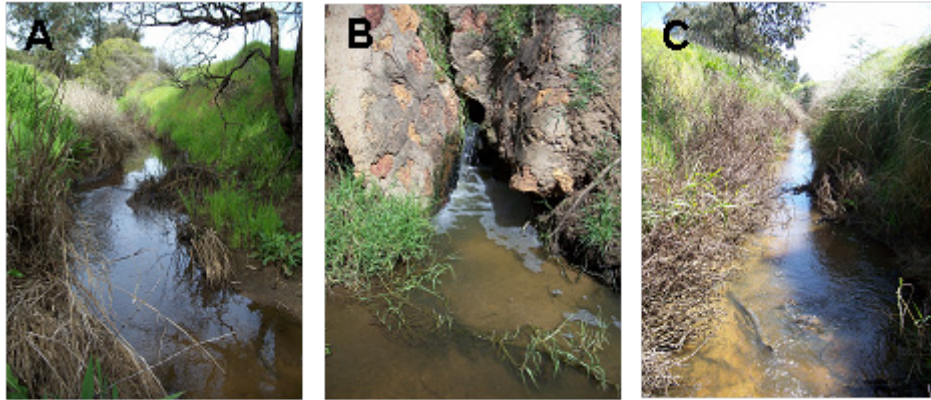


Figure 14: Sampling locations at Waroona WWTP

Notes: A. Upstream of WWTP; B. Waroona WWTP discharge point; C. Downstream of Waroona WWTP.



Figure 15: Sampling site on the Serpentine River directly east of the entrance to Serpentine National Park

2.4.2 Morphological measurements

Fish were removed from the freezer and thawed to room temperature. All fish were examined under a dissection microscope and categorised as adult male, immature male, adult female and juvenile female. Adult male fish were defined as having a fully developed gonopodium with a terminal complex of hooks (Figure 16) and immature males as having evidence of gonopodial development (either elongation or thickening of the third gonopodial ray) but no terminal complex of hooks. Female fish were identified as phenotypically adult if the split in the third ray of the anal fin extended to within three ray segments of the body. Gravid females were also identified based on the presence of a black spot on the abdomen. Counts were made of each group to establish a sex ratio and a measure of the proportion of males that had a fully developed gonopodium (mature males).

Morphological measurements were conducted on both morphologically mature and morphologically immature male fish and included (Figure 16):

- standard length (SL) – the distance from the snout to the caudal plate
- gonopodial length (GL) – the length of the fourth ray of the gonopodium
- gonopodial extension (Gx) – the distance that the fourth ray of the gonopodium extends beyond the sixth ray of the gonopodium
- pre-anal length (PAL) – the perpendicular distance from the point of attachment of the gonopodium to the snout of the fish
- wet weight (WW) – the mass of the fish having been blotted dry but not oven dried.

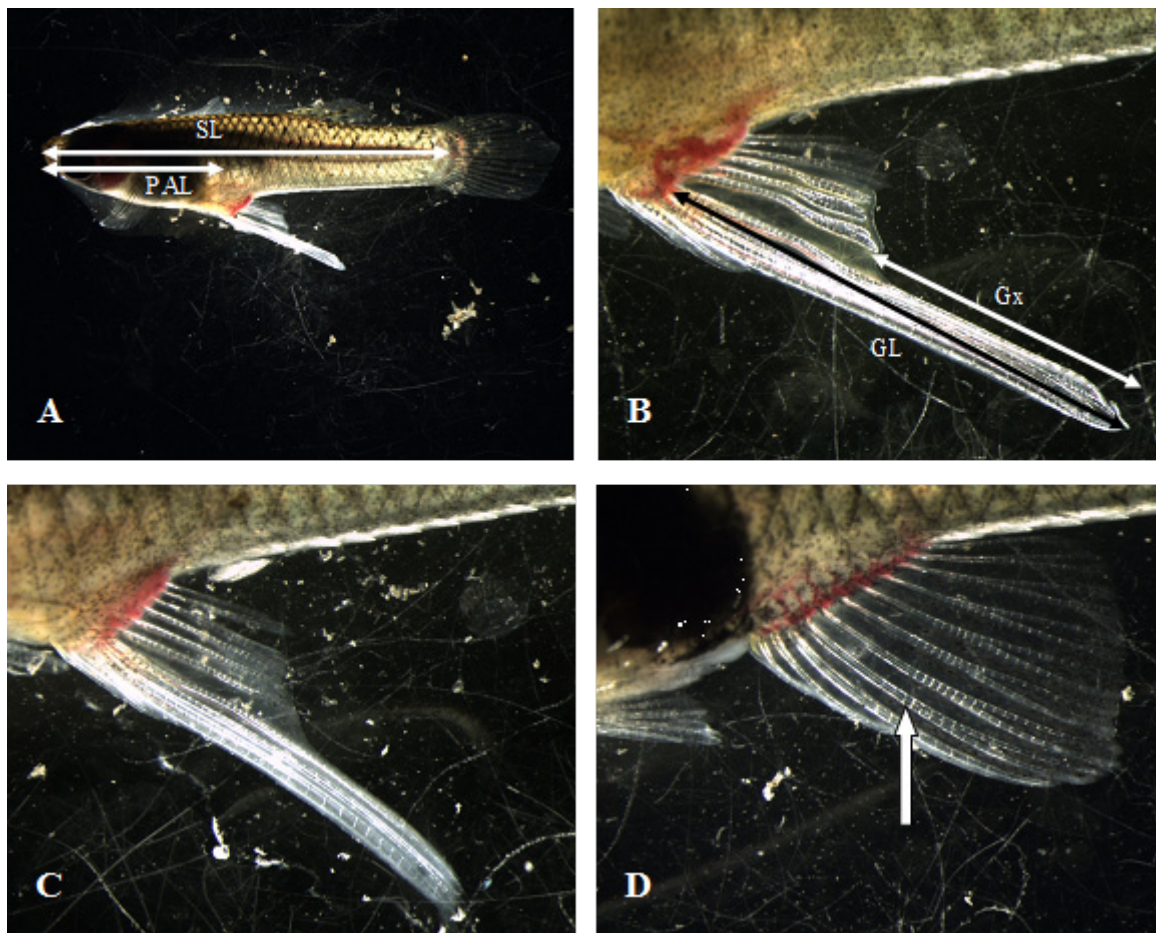


Figure 16: Morphological measurements made on *Gambusia holbrooki* captured from the study sites

Notes: A. Standard length (SL) and pre-anal length (PAL); B. Gonopodial length (GL) and gonopodial extension (Gx); C. Male with gonopodial development but no terminal complex of hooks (as seen in B); D. Normal female anal fin with no elongation (note the split in the third ray extending to within three ray segments of the point of attachment indicated by white arrow).

These measures enable an analysis of the degree of gonopodial development and a calculation of the condition of the fish (Fulton's $K = \text{weight}/ \text{length}^3$). In particular the measures of GL, Gx, PAL, sex ratio and the proportion of males that are morphologically mature can be used to establish exposure to estrogenic or

androgenic EDCs (see Table 6 for expected effects of EDCs on measured endpoints). Morphological measurements were made using a digital micrometer under a dissection microscope (mag. x 40).

Table 6: Male mosquitofish reproductive endpoints and population measures used to indicate the exposure to estrogenic and androgenic EDCs and the expected impact on each measure

		Under estrogenic impact	Under androgenic impact
Gonopodial length	(GL)	-	+
Gonopodial extension	(Gx)	-	+
Pre-anal length	(PAL)	+	-
Proportion of phenotypic males in the population	(sex ratio)	-	+
Proportion of males with hooks		-	no change

Notes: Androgenic impact is assessed taking the size of the fish into account and is a proposed result requiring empirical confirmation. + indicates an increase in the metric; - indicates a decrease in the metric. nd = no data available.

2.4.3 Vitellogenin measurement

Vitellogenin was quantified in fish samples taken from each site in summer (March 2009) and winter (July 2009). Where possible 20 adult male fish (with developed gonopodia) and six adult female fish (large) from each sampling site in winter and summer were individually homogenised in 1:4 (w:v) Tris buffer (3 mM Tris, 0.1 µM PMSF) for 30 seconds immediately following morphological measurements. The homogenates were centrifuged at 8000 x g for 40 minutes at 4°C. The supernatant was removed and frozen at -80°C until the bioassay was conducted. Vitellogenin content of the supernatant was semi-quantitatively assessed by enzyme linked immunosorbent assay (ELISA).

Mosquitofish homogenate supernatants were diluted (1:1000) in a carbonate/bicarbonate coating buffer (50 mM, pH 9.6) as was a salmonid vitellogenin standard (Sapphire Biosciences). The layout of the plates was determined by random number generation ensuring no plate or row bias. 100 µL of each sample and standard were added to the wells of a clear ELISA plate (Iwaki) and allowed to incubate for 24 hours at 4°C. After this incubation period the wells were washed (x 3) in a Bio-Rad plate washer with (300 µL) phosphate buffered saline (0.01 M, pH 7.3) with 0.05% Tween. A blocking solution (1% bovine serum albumin in phosphate buffered saline) was added to each well (200 µL). The plate was then incubated and the wells emptied. The monoclonal antibody BN-5 (Sapphire Biosciences) was diluted (1:100) in the blocking solution, added to the wells (100 µL) and incubated for one hour at 37°C. The plates were again washed (x 3) and a secondary antibody (goat anti-rabbit horseradish peroxidase (HRP) conjugate; Sapphire Biosciences)

was diluted (1:2000) in the blocking solution and added to the wells (100 μ L) for a further hour at room temperature. After a further five wash cycles a peroxidase substrate (OPD fast; Sigma) was added to the wells (100 μ L) for a 15-minute dark incubation. To stop the development reaction 2M H₂SO₄ (50 μ L) was added to the wells and the absorbance read immediately at 490 nm.

This semi-quantitative analysis for vitellogenin content allows comparative measures (e.g. between sites and sampling periods or against a reference condition) to be made but does not give absolute values for the protein. Thus all samples were analysed in a single run of the assay. This is essential in a semi-quantitative analysis to ensure comparability between results. The vitellogenin content (as semi-quantified by the ELISA) was standardised against the protein content of the supernatant (calculated by the method of Lowry et al. 1951).

2.5 Data analysis

2.5.1 Predicted biological activity

In vitro activity could be predicted for estrogenicity, androgenicity and photosynthesis inhibition. Cytotoxicity and genotoxicity can be caused by a wide variety of compounds, and it was therefore not possible to relate these biological endpoints to chemical analysis.

Predicted estrogenicity was calculated based on the concentrations of 17 β -estradiol, 17 α -estradiol, estrone, estriol and 17 α -ethynylestradiol determined by chemical analysis multiplied by their relative potency in the E-SCREEN bioassay. The relative potencies used in this report were: 17 β -estradiol = 1; 17 α -estradiol = 0.1; estrone = 0.05; estriol = 0.17; and 17 α -ethynylestradiol = 1.06.

Predicted androgenicity was calculated based on the concentrations of androstenedione, androsterone, etiocholanolone, testosterone, dihydrotestosterone, and testosterone propionate determined by chemical analysis multiplied by their relative potencies in the AR-CALUX bioassay. The relative potencies used in this report were: androstenedione = 0.0572; androsterone = 0.0056; etiocholanolone = <0.0001; testosterone = 0.2135; dihydrotestosterone = 1; testosterone propionate = 0.1998 (Houtman et al. 2009).

The predicted diuron equivalent (DEq) was calculated based on the concentration of diuron as determined by chemical analysis.

2.5.2 *In vitro* bioassays data analysis

2.5.2.1 *MicroLumo*

The results are expressed as a toxic unit (TU_{WR}) calculated as 1/EC₅₀ of the sample, where the EC₅₀ is expressed in mL of the original water sampled.

The Microtox assay (on which the MicroLumo assay is based) is often recommended as a screening tool for effluent quality monitoring (Harsham 1995) and the United States Environmental Protection Agency (USEPA) is currently considering incorporating the assay for regulation (USEPA 2007). Using the standard 'Microtox

81.9% base test method', an effluent would be declared non-compliant if it exceeded 50% toxicity. Converting this 81.9% to the MicroLumo, this would be equivalent to $150 * 0.819 = 123$ uL. For the assay used here, the reference toxic unit in water would be:

$$TU_{WR} = 1 / 0.123 = 8.1.$$

Any value above a TU of 8.1 would then be above the proposed guideline; any value below this would be considered as compliant.

2.5.2.2 *umuC* assay

Samples are considered genotoxic when the induction ratio of the sample exceeds 1.5 and the growth rate exceeds 0.5.

$$\text{Induction ratio (IR)} = \frac{\beta\text{-Galactosidase activity}}{\text{Bacterial growth}} \quad \text{Equation 1}$$

The values presented in this report are interpolated 1.5 induction ratio values derived from a linear regression using Graph Pad Prism in mL of original water. The 1.5 induction value is then converted to a genotoxic unit (1/1.5 induction ratio in mL water) for consistency with other assays. Values reported as not genotoxic were below the 1.5 induction ratio at the highest allowable sample application volume (5 uL solvent, 1.9% solvent by volume).

While there is no guideline for genotoxicity, a water sample would be considered genotoxic if it resulted in an induction of 1.5 without SPE enrichment. In this bioassay, this would be equivalent to $1/0.180 = 5.5$ genotoxic units (GTUs). In other words, any water sample that exceeds 5.5 GTUs would be considered genotoxic, while a sample below that value would have to be artificially concentrated to be genotoxic.

2.5.2.3 *Max-I-PAM*

The wastewater sample results proved to be challenging to interpret as there were significant cytotoxic effects, and 'non typical' dose response curves. In an effort to characterise the effects, three time points were measured (30 minutes, two hours and 24 hours). A typical dose response from a herbicide will have a similar photosynthetic inhibition effect curve for all time points and a slope approaching 1 (see Figure 17). Samples dominated by herbicides will have similar dose response curves over time, especially at the 30-minute and two-hour time points; samples dominated by non-specific cytotoxic compounds will have an increasing inhibition of yield over time and a steep slope. Therefore, where a sample is classified as 'herbicidal' in the following data, it means the sample dose response at two hours was similar to, or the same as, the 30-minute response with a slope approaching 1. A sample described as cytotoxic will typically have an increasing inhibition of yield over time and a steep slope (>2).

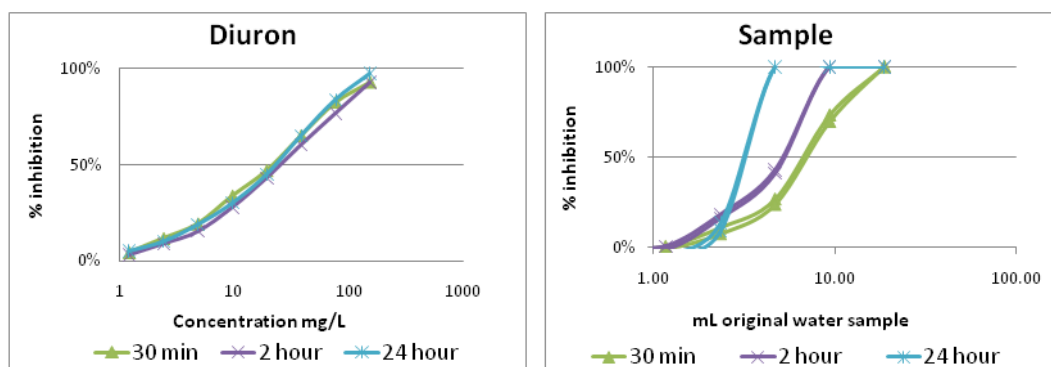


Figure 17: Typical Max-I-PAM response curves

Notes: Left: Typical herbicide-dominated dose response effect over three time periods: 0.5, 2 and 24 hours. This specific curve is from the positive-control PSII herbicide diuron. Right: Example of a sample with increasing toxicity over time, possibly due to non-specific cytotoxic effects.

2.5.2.4 E-SCREEN

The E-SCREEN results are expressed as estradiol equivalents (EEq). The EC50 values of the E-SCREEN response (i.e. cell proliferation) derived from a 17β -estradiol standard curve (in ng/well) was divided by the EC50 value obtained from a sample dilution series (in L equivalent/well) to obtain the EEq (in ng/L).

2.5.2.5 AR-CALUX

The AR-CALUX results are expressed as dihydrotestosterone equivalents (DHT Eq). The EC50 values of the AR-CALUX response (i.e. luciferase induction) derived from a dihydrotestosterone standard curve (in ng/well) was divided by the EC50 value obtained from a sample dilution series (in L equivalent/well) to obtain the DHT Eq (in ng/L).

2.5.3 *In situ* analysis

For *in situ* measurements of the male mosquitofish, differences in GL and PAL were analysed using single factor analysis of covariance (ANCOVA) with the length of the fish as a covariate. The assumptions of homogeneous covariate slopes and equal variances between groups were satisfied. Sex ratios were examined using a binomial test. All statistical analyses were conducted using the SPSS statistical software package (SPSS, v 17.0).

3 Results and discussion

3.1 Chemical analysis

3.1.1 Hormones

The results of the chemical analysis for estrogenic and androgenic steroids are presented as lognormal probability plots (figures 21 to 32). These plots show the variability of the different sample types throughout the sampling period. While the available data are limited, these plots do provide a clear visualisation of the following attributes:

- concentration region (range) of the available data
- degree of variability (as indicated by the slope of the line)
- degree of removal between subsequent sample types (i.e. treatment efficiency)
- limits of reporting (LOR) and the proportion of data below the LOR.

Several androgenic and estrogenic hormones were detected in raw and partially-treated wastewater (figures 18, 19 and 20). Some androgens were commonly measurable above the LOR (particularly androstenedione, androsterone, eticholanolone and testosterone), and only one estrogen (estrone) was commonly above the LOR.

Three hormones (dihydrotestosterone, 17 α -estradiol and 17 α -ethynylestradiol) were not detected in any of the samples (the LOR for these compounds are 50, 5 and 5 ng/L respectively).

Overall, the chemical data indicated good removal during secondary treatment and, with a few exceptions, hormones were not detected in treated wastewater. The exceptions were low concentrations of etiocholanolone in the Beenyup AWTP secondary effluent and the Harvey and Gordon Road WWTP effluents (Figure 18C). Estrone was detected in treated effluent from all plants (Figure 20B), and estriol was found in the Beenyup AWTP secondary effluent, and Waroona and Harvey WWTP final effluents (Figure 20C).

None of the monitored chemicals were detected in RO permeate.

There are only a few published reports regarding the concentrations of steroidal hormones in Australian WWTPs (Chapman 2003; Leusch et al. 2005a; Leusch et al. 2006c; Tan et al. 2007b; Coleman et al. 2009; Braga et al. 2005b) However, the available data indicates that concentrations and patterns of estrogenic and androgenic hormones in Australian wastewater are similar to those reported in other countries. Furthermore, the concentrations and observed removal during treatment for the AWTP and WWTPs investigated in this study are consistent with previous reports from Australia and overseas.

A literature review examining a range of international studies revealed that adsorption onto suspended solids (organic and colloidal), aerobic and anaerobic

biodegradation, abiotic chemical degradation (e.g. hydrolysis) and volatilisation are the key removal mechanisms for steroidal estrogens during wastewater treatment processes (de Mes et al. 2005).

The only unusual finding was the observation of the androgenic steroid trenbolone in raw influents and primary WWTP effluents (although it was not observed in more highly treated effluents). No other studies are known in which this chemical has been determined in municipal wastewaters.

The following discussion of estrogens and androgens in municipal wastewaters is provided to place the current results from the four Western Australian treatment plants in the context of earlier data and existing knowledge on the presence and removal of these chemicals.

3.1.1.1 Estrogens

Two estrogens, 17α -estradiol and ethinylestradiol, were below the LOR (<5 ng/L) in all samples of all four treatment plants. 17β -estradiol was also commonly below the LOR (<5 ng/L), but was occasionally identified in raw influent and primary effluents at Beenyup AWTP and Gordon Road WWTP, as well as once in secondary effluent at Gordon Road WWTP. On the other hand, estrone and estriol were commonly identified (in the range 5 – 1000 ng/L) in influents and effluents from the four treatment plants.

The natural estrogens 17β -estradiol, 17α -estradiol, estrone and estriol are excreted by humans, either unconjugated or primarily as inactive glucuronide or sulfate conjugates; however, these conjugates can be rapidly cleaved and metabolised into their active steroidal forms by enzymes present in wastewaters. The low concentrations of 17β -estradiol and 17α -estradiol in the samples have previously been explained by rapid degradation of the estradiols to estrone (Servos et al. 2005). 17β -estradiol was also undetected in previous studies (Lee et al. 2005; Tan et al. 2008) or was found in a similarly low ng L⁻¹ range as reported here (Hu et al. 2007) with a maximum concentration of 16 ng L⁻¹ (Braga et al. 2005b).

Batch experiments by Ternes et al. (1999a) have confirmed that 17β -estradiol is degraded rapidly and easily to estrone. There are no known reports of 17α -estradiol being present in Australian wastewaters. The main active compound of the contraceptive pill, ethinylestradiol, has also been undetectable in some previous studies (Snyder et al. 2007; Tan et al. 2008) or if detected, the concentrations were again in the very low ng/L range. The occurrence of estriol in wastewater has been reported in several studies at up to 318 ng/L (Kim et al. 2007), whereas it was undetectable in others (Snyder et al. 2007). Estrone is generally – apart from the less potent estriol – the most abundant steroidal estrogen in wastewater, with concentrations in the range of 15–54 ng/L reported in final effluents (Braga et al. 2005c; Ternes et al. 1999a; Tan et al. 2008).

According to Johnson and Sumpter (2001), the most likely pathways for estrone to occur in wastewater are the degradation of its glucuronide and sulfonide conjugates and of 17β -estradiol in the sewer system. Due to its moderately high estrogenic potency and its role as a key metabolite of estradiol and other estrogenic hormones,

estrone has been identified as the most environmentally important estrogen (Johnson & Sumpter 2001). However, 17β -estradiol and ethinylestradiol can also play minor but still important roles in the overall estrogenic activity of wastewaters. Estriol appears to give less cause for environmental concern, since it is generally released in low concentrations into the environment and shows a relatively low potency compared with other steroidal hormones (Johnson & Sumpter 2001).

Reported removal rates of estrone for conventional activated sludge plants generally range from 64% (Lee et al. 2005) to 85% (Braga et al. 2005b). Negative removal efficiencies for estrone have been reported in previous studies (Carballa et al. 2004), which were believed to be caused by further degradation of 17β -estradiol and cleavage of glucuronide and sulfonide conjugates of estrone during wastewater treatment.

Leusch et al. (2005a) reported between 92 to 99% removal of estrogenic activity following activated sludge treatment, and Andersen et al. (2003) reported 90 to 98% removal efficiencies for natural and synthetic estrogens in German WWTPs, mostly as a result of activated sludge treatment. Coleman et al. (2009) investigated several WWTPs in New South Wales for removal of estrogenic activity and reported removal rates of up to 99% for activated sludge processes and 87% for a membrane bioreactor process. Servos et al. (2005) also reported that well-operated WWTPs with adequate levels of nitrification tended to have higher removal of estrogens than those that did not nitrify; however, it remained unclear whether this was due to co-metabolism by nitrifying microbes or was simply a reflection of an enhanced biotic diversity and better growth conditions in these well-functioning WWTPs. It was also likely to be a result of greater microbial biomass and activated sludge bulk produced in effectively-functioning activated plants, leading to more sites for adsorption of steroidal hormones and physical removal in subsequent clarification processes.

3.1.1.2 Androgens

All of the androgens investigated in this project were detected at some point, except the testosterone metabolite dihydrotestosterone (DHT). The failure to observe DHT may, in part, be due to the relatively high limit of reporting for this analyte (50 ng/L). No previous reports of DHT in wastewater are known. As suggested by Kirk et al. (2002), the main pathway for androgens entering domestic wastewater networks is most likely through human excretion. Concentrations of androgens in wastewater are expected to be much higher than for estrogens due to their higher excretion rates in humans. However, results from previous investigations (Kim et al. 2007; Snyder et al. 2007) suggest that androgens are removed effectively during secondary treatment, and consistent results were generally observed in the present study.

It is interesting to note that trenbolone was detected in the raw influents of the WWTPs and also in the primary effluent at Beenyup AWTP (but not in the secondary effluents). Trenbolone is a metabolite of trenbolone acetate, which is administered to beef cattle as a growth promoter (Khan et al. 2008). To our knowledge, this is the first report of trenbolone being detected in a WWTP.

Although the presence of androgens in wastewater has been known for some time (Shore et al. 1993), information about the effectiveness of various treatment processes on attenuating the androgenic activity of effluents is, alongside that of estrogens, comparatively limited (Kirk et al. 2002; Svenson & Allard 2004; Tan et al. 2007b; Coleman et al. 2009; Leusch et al. 2006c).

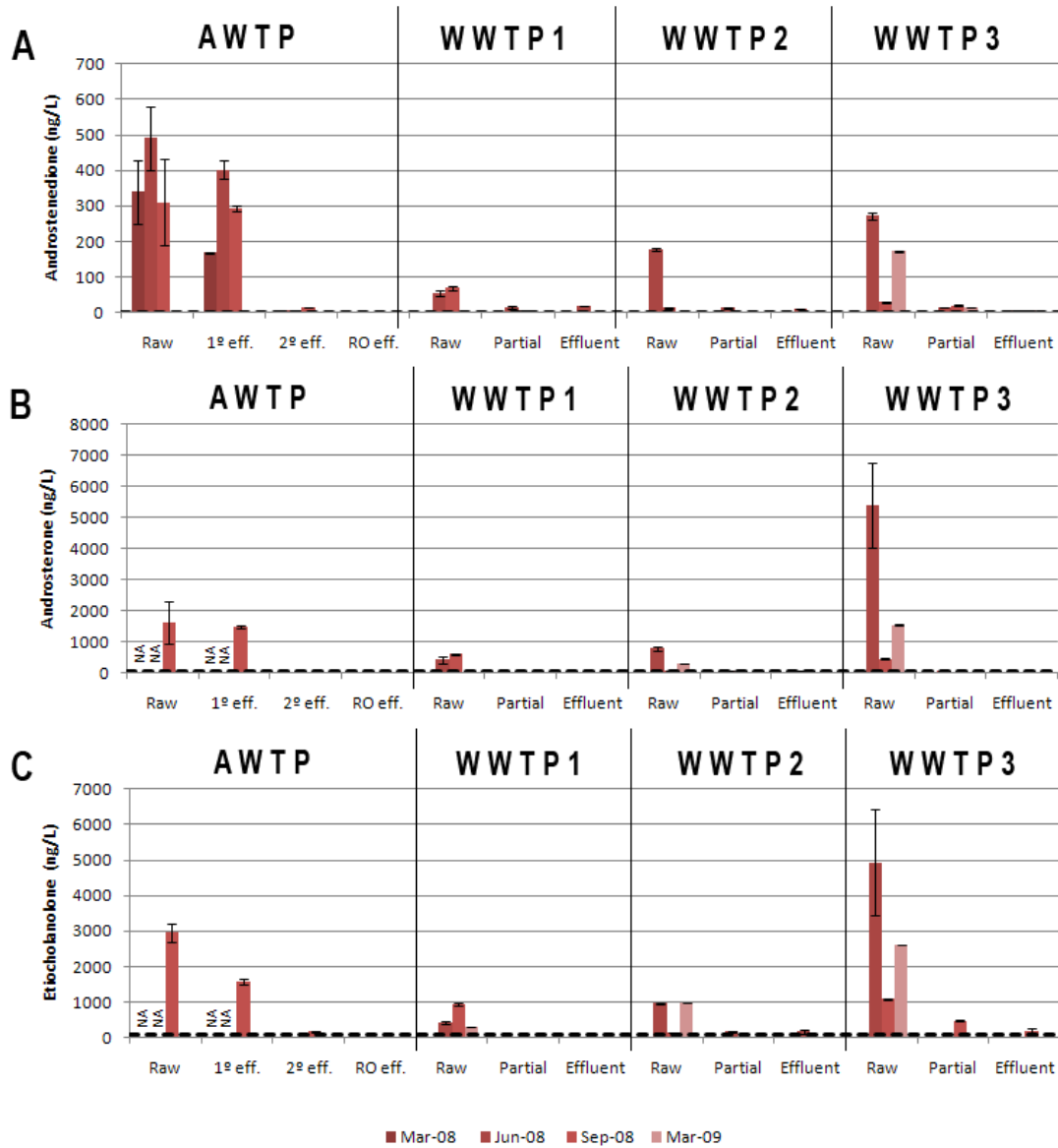


Figure 18: Concentration of androgens at the four plants (one of two)

Notes: (A) androstenedione, (B) androsterone and (C) etiocholanolone in samples from Beenyup (AWTP), Waroona (WWTP1), Harvey (WWTP2) and Gordon Road (WWTP3), in ng/L. The dashed line represents the limit of reporting. NA = not available.

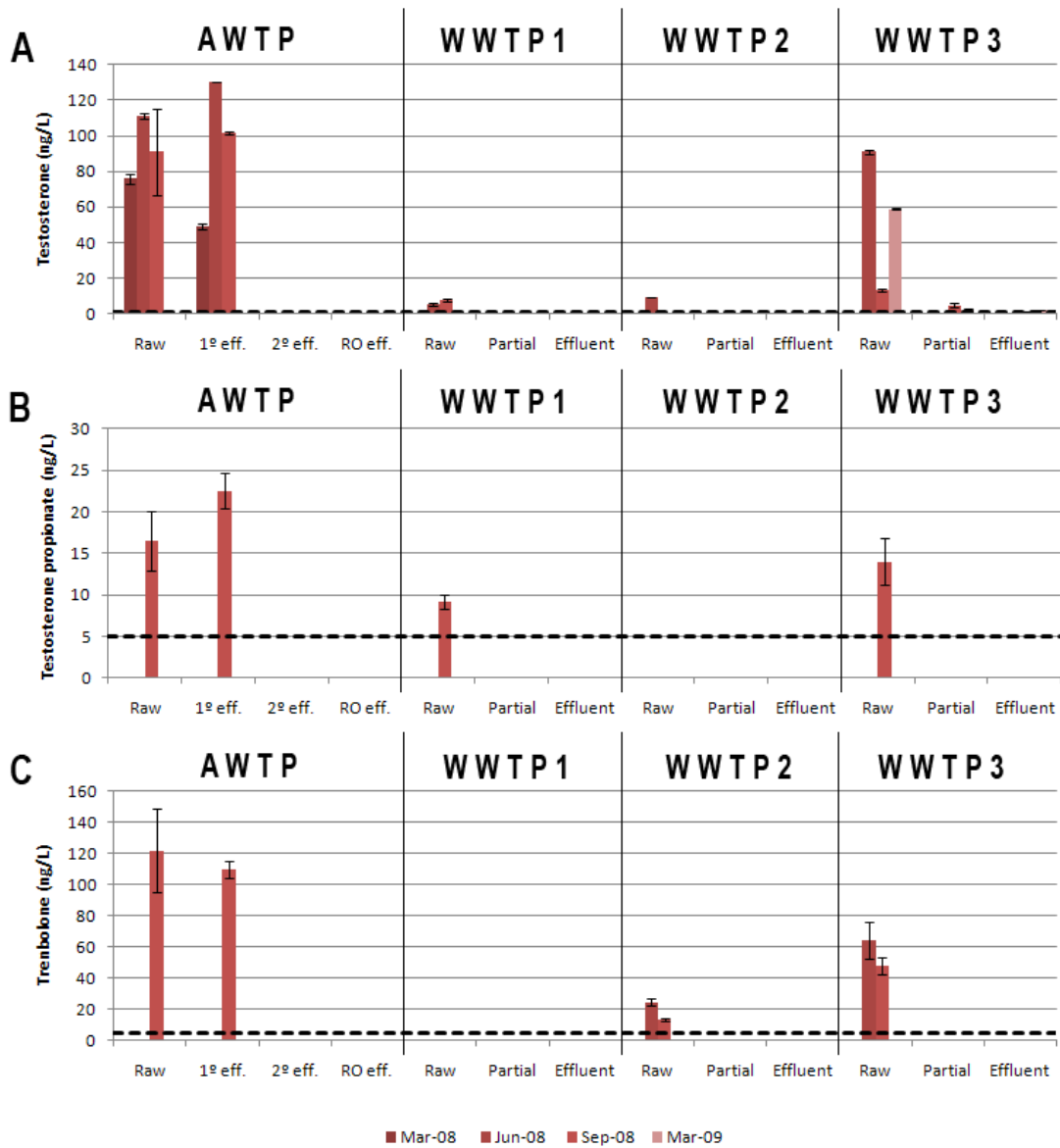


Figure 19: Concentration of androgens at the four plants (two of two).

Notes: (A) testosterone, (B) testosterone propionate and (C) trenbolone in samples from Beenyup (AWTP), Waroona (WWTP1), Harvey (WWTP2) and Gordon Road (WWTP3), in ng/L. The dashed line represents the limit of reporting. NA = not available.

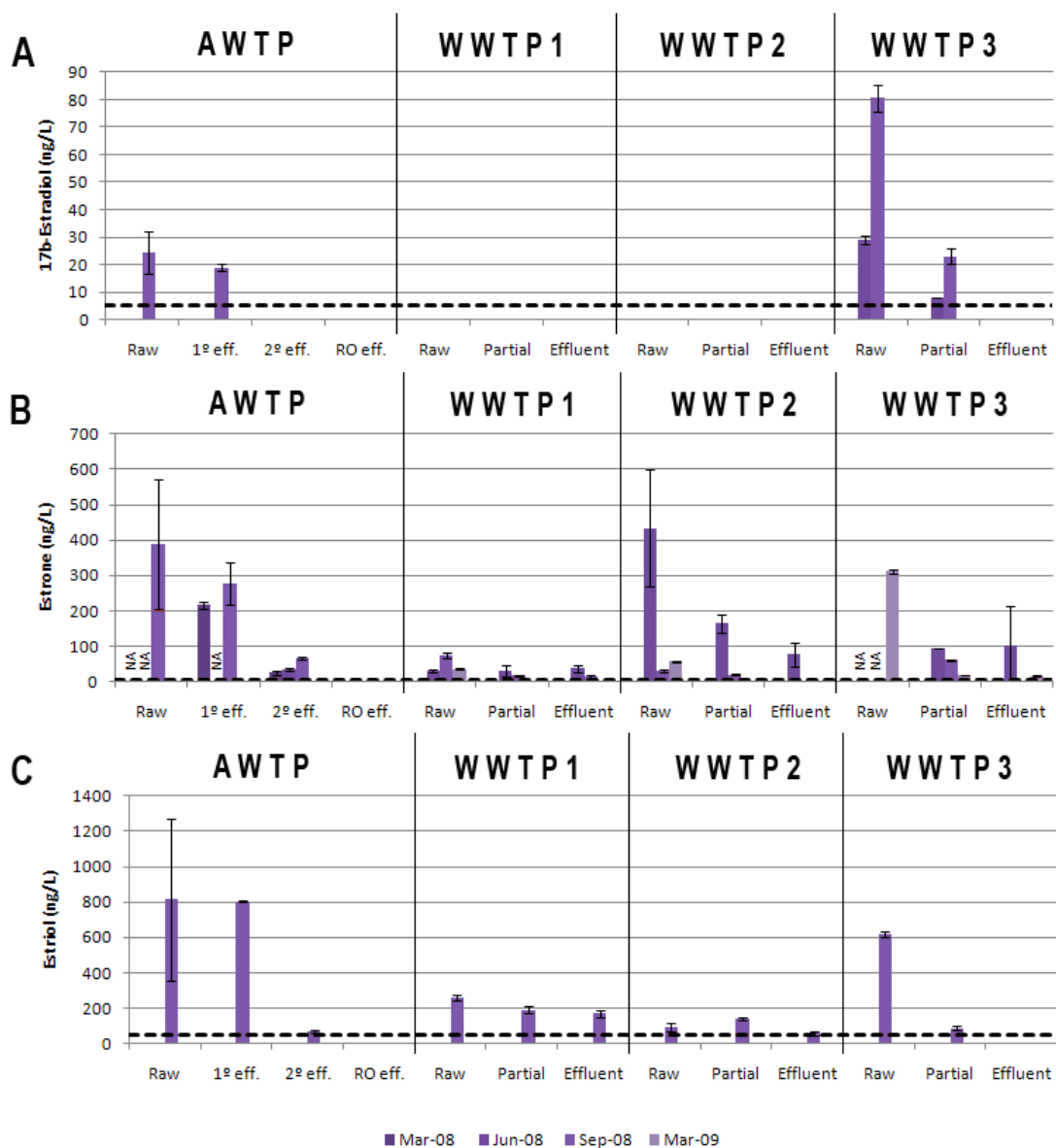


Figure 20: Concentration of estrogens at the four plants

Notes: (A) 17 β -estradiol, (B) estrone and (C) estriol in samples from Beenyup (AWTP), Waroona (WWTP1), Harvey (WWTP2) and Gordon Road (WWTP3), in ng/L. The dashed line represents the limit of reporting. NA = not available.

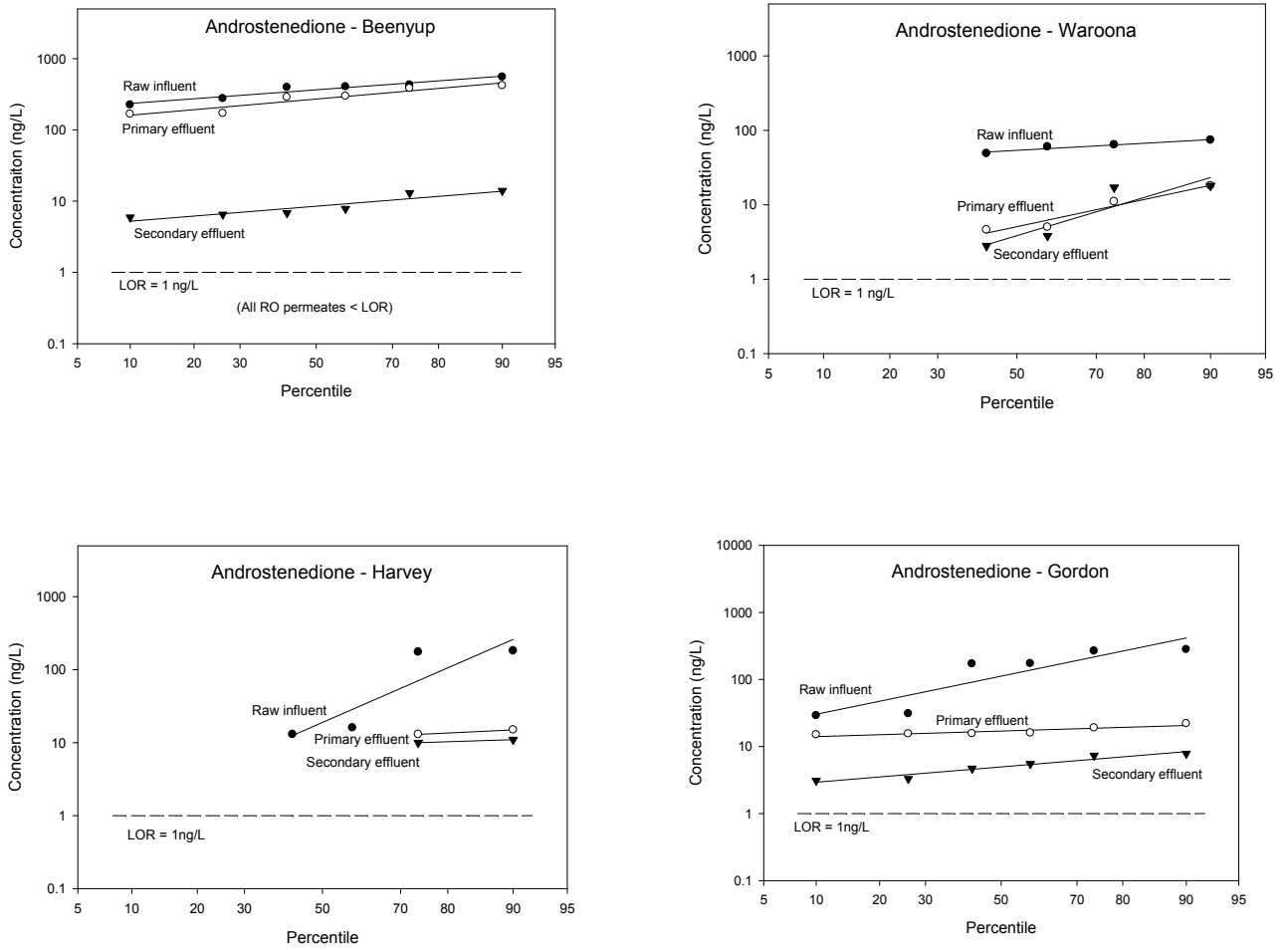


Figure 21: Androstenedione concentration at the four plants

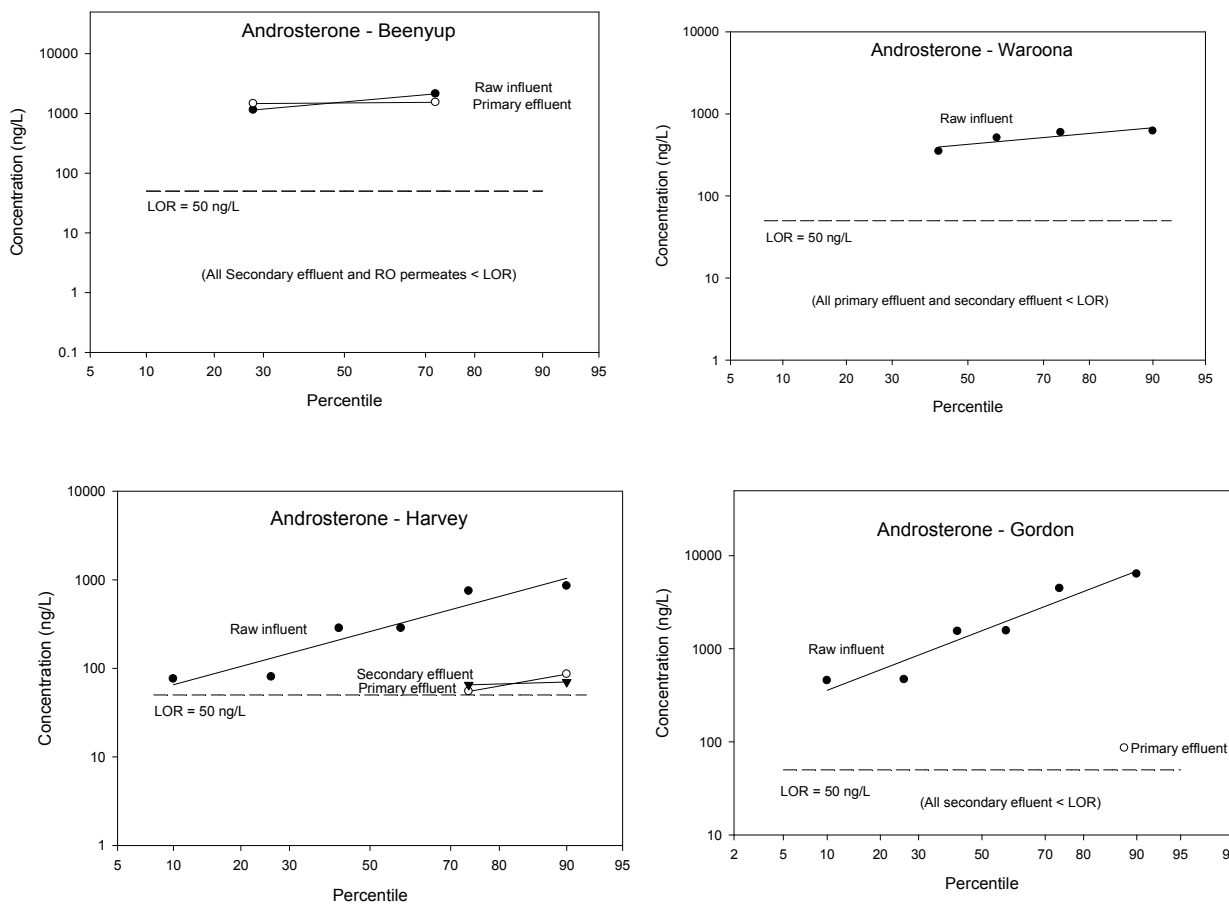


Figure 22: Androsterone concentration at the four plants

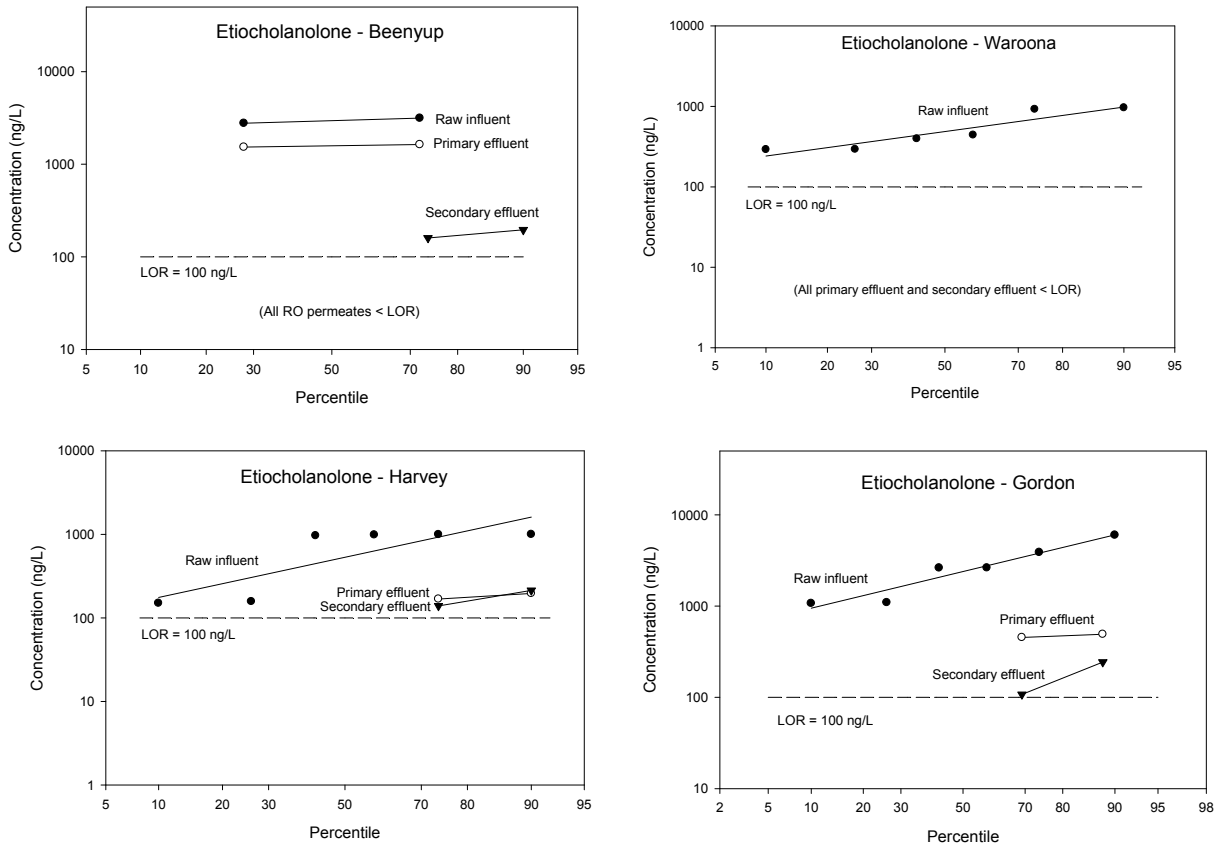


Figure 23: Etiocholanolone concentration at the four plants

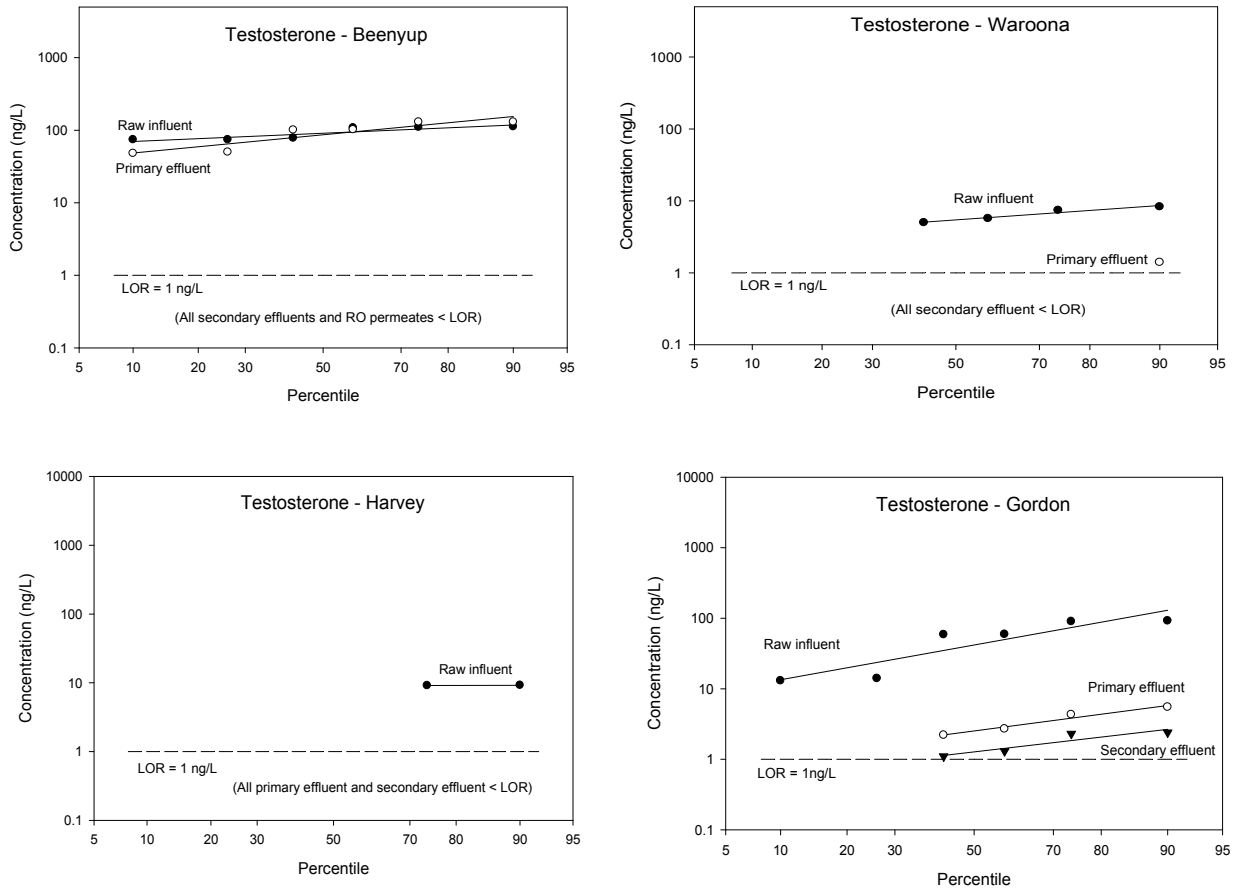


Figure 24: Testosterone concentration at the four plants

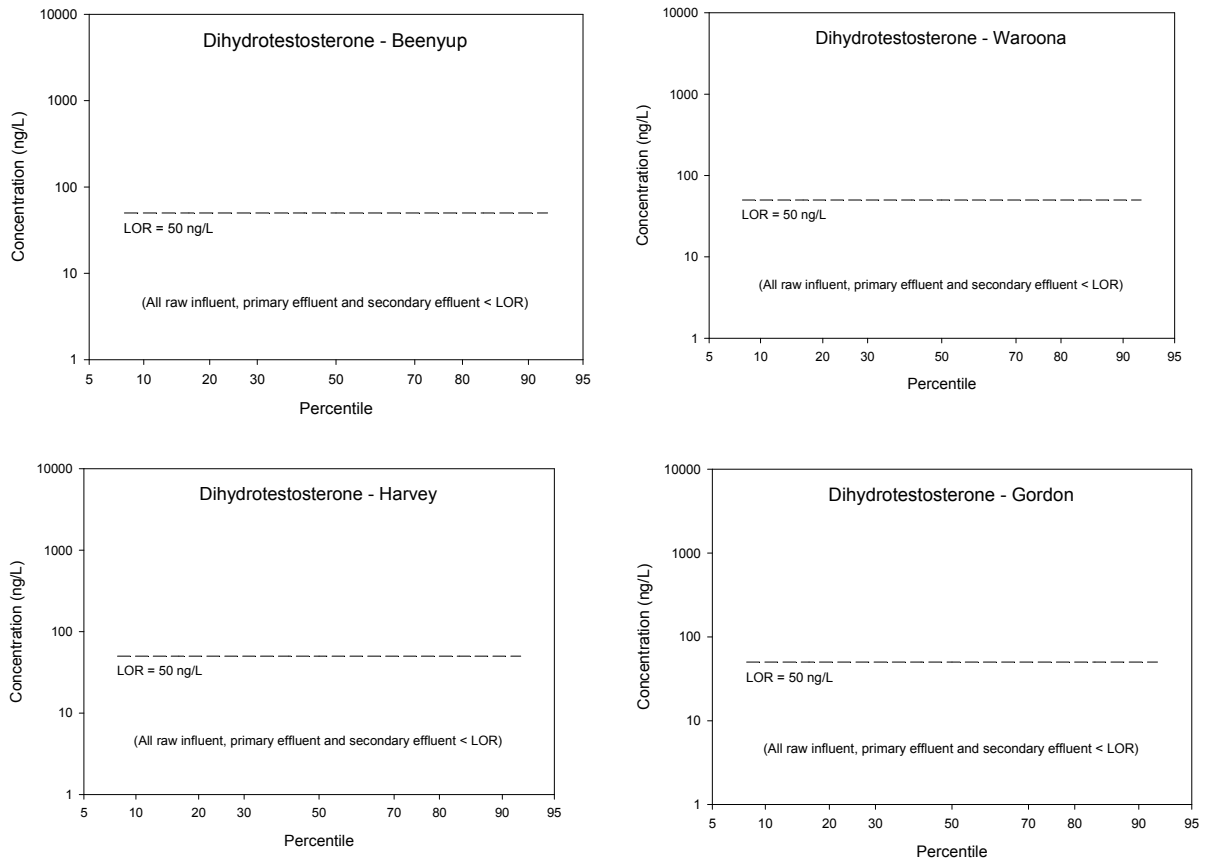


Figure 25: Dihydrotestosterone concentration at the four plants

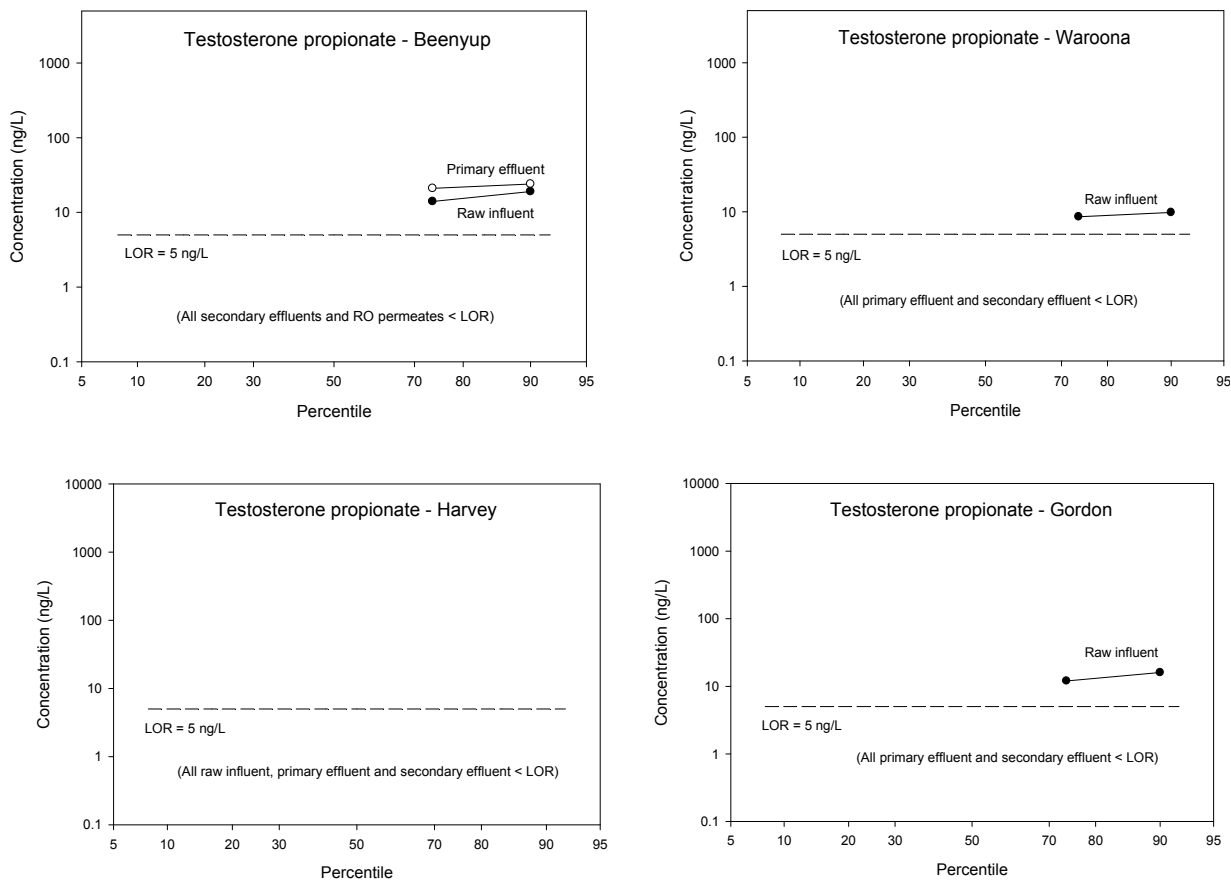


Figure 26: Testosterone propionate concentration at the four plants

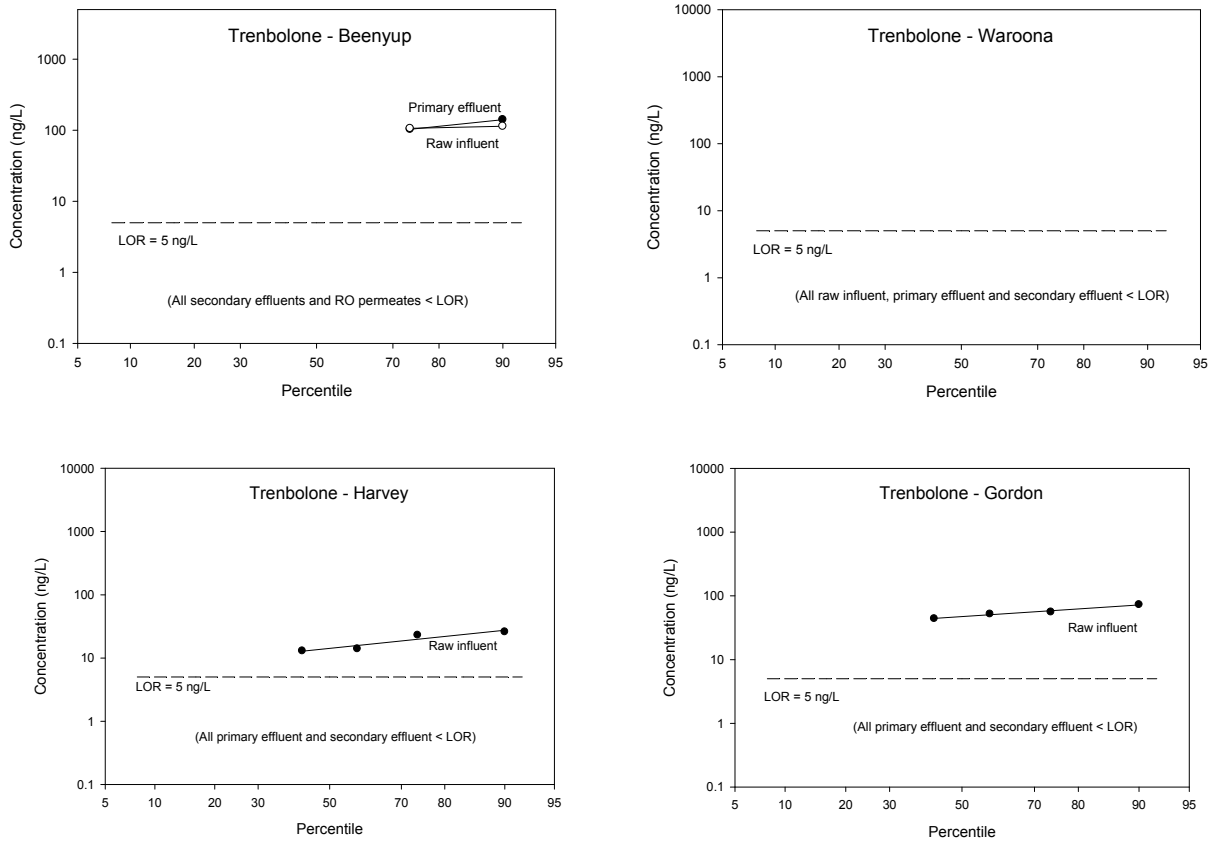


Figure 27: Trenbolone concentration at the four plants

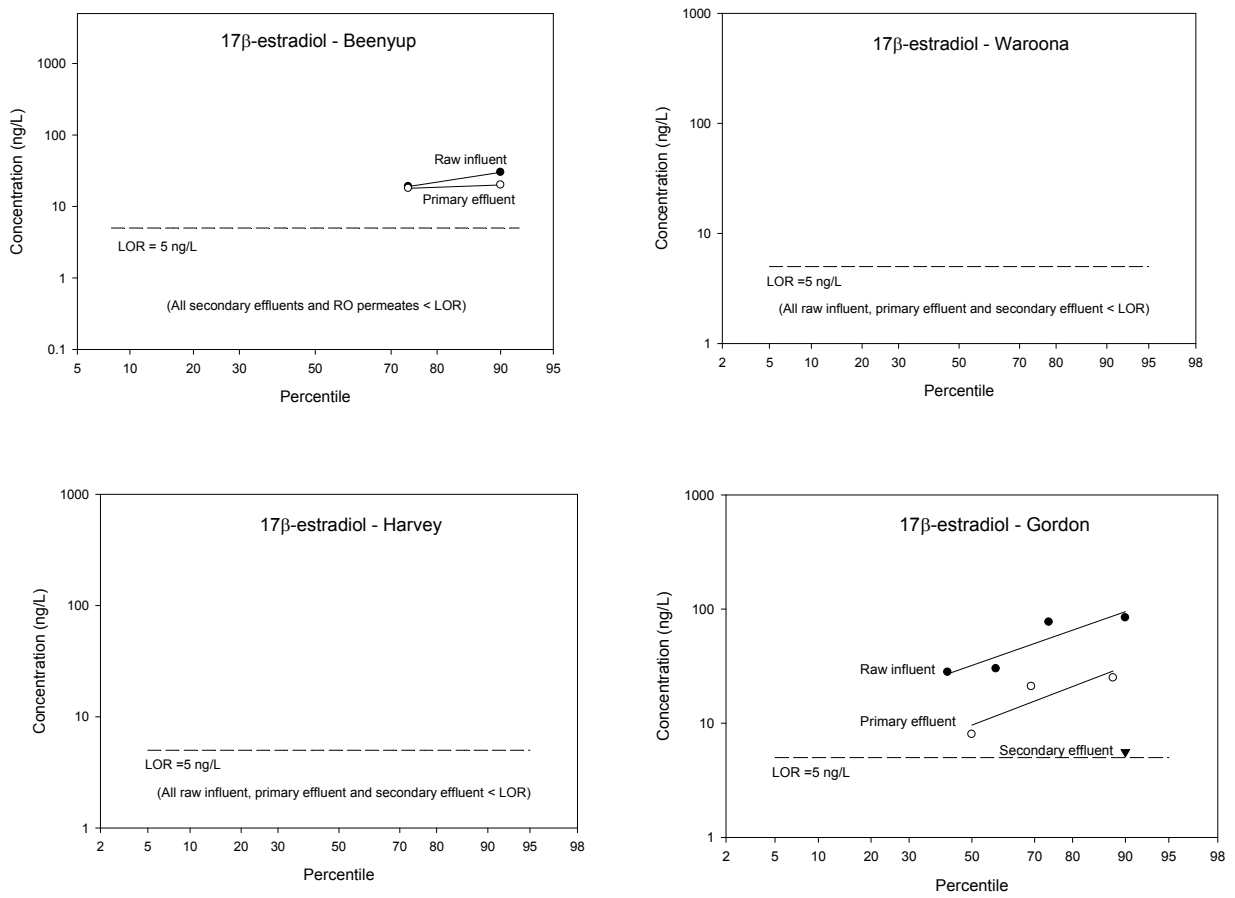


Figure 28: 17β-estradiol concentration at the four plants

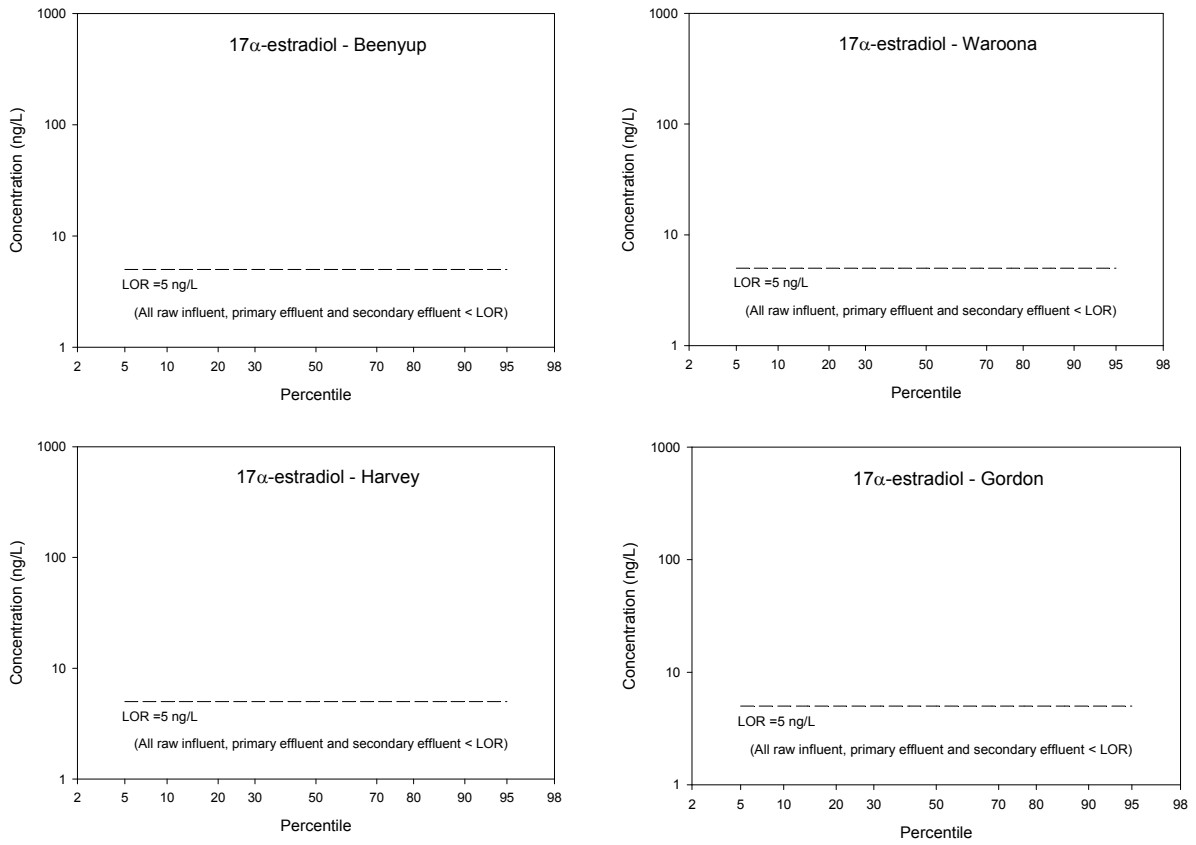


Figure 29: 17α-estradiol concentration at the four plants

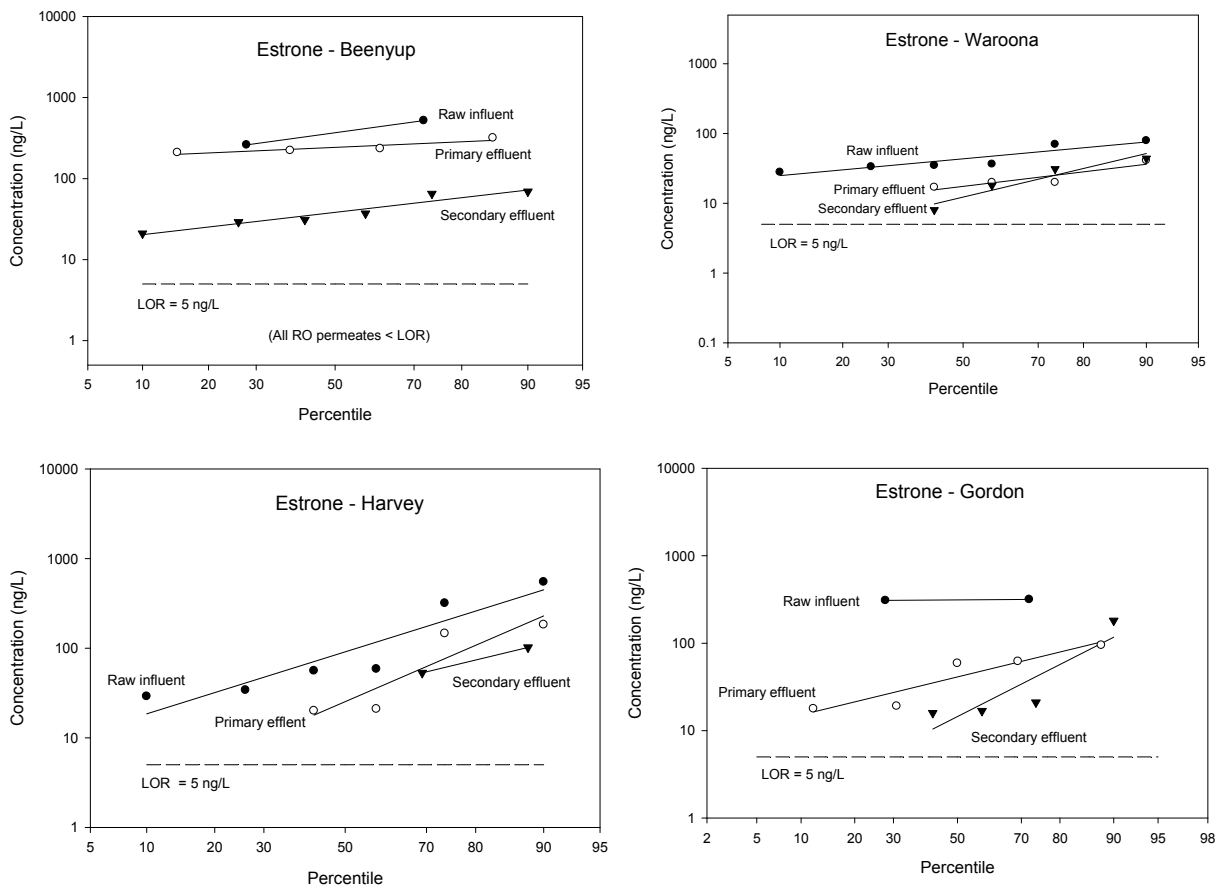


Figure 30: Estrone concentration at the four plants

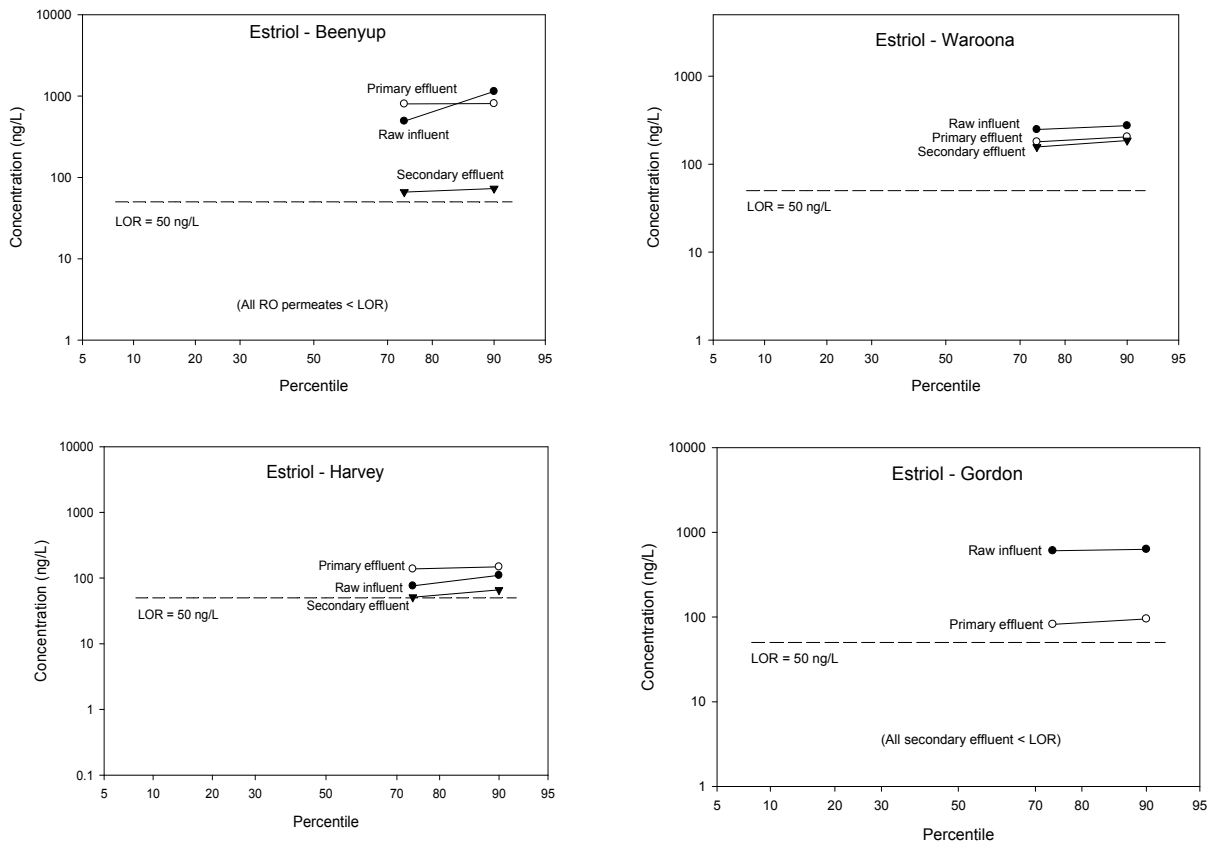


Figure 31: Estriol concentration at the four plants

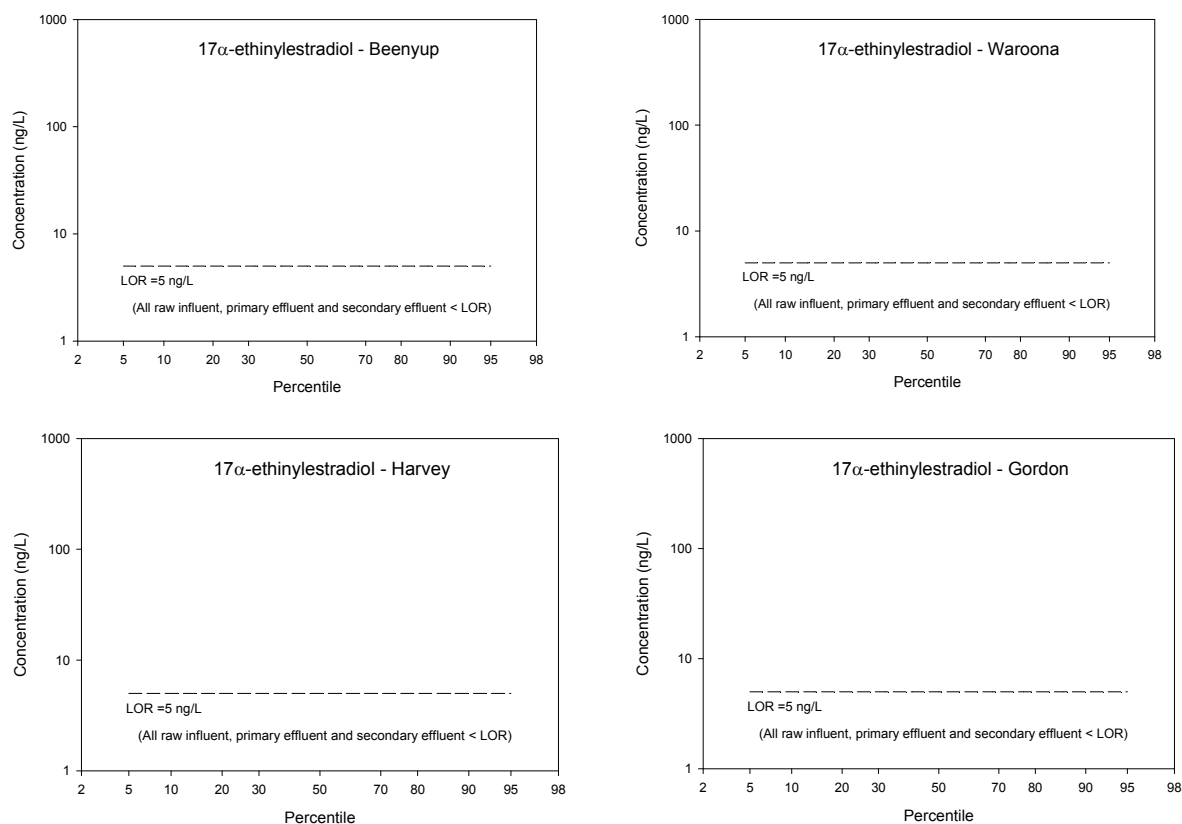


Figure 32: 17α-ethinylestradiol concentration in the four plants

3.1.2 Herbicides

Of all the pesticides monitored (Section 2.2.2), only diuron and metolachlor were detected above the reporting limit ($>0.025 \mu\text{g/L}$) (Figure 33).

Diuron was detected at most WWTPs, with concentrations ranging from <0.025 to $0.15 \mu\text{g/L}$. The regulatory levels for diuron in the ANZECC guidelines are $0.2 \mu\text{g/L}$ (low reliability) for freshwater ecosystems, $2 \mu\text{g/L}$ in water intended for irrigation and $40 \mu\text{g/L}$ in water intended for recreational purposes (2000). The *Australian guidelines for water recycling 2008 Phase 2* recommends a human health guideline of $30 \mu\text{g/L}$ for diuron. The concentrations reported here are always below any of these guideline values.

Treatment did not appear to significantly affect the concentration of diuron in the water, and effluent water was usually in the same range as the influent. RO was, however, effective at removing diuron to below quantification level ($<0.025 \mu\text{g/L}$) (AWTP, Figure 33A).

Metolachlor was detected at the Beenyup AWTP only (Figure 33B). The concentration in the influent ranged from just above the reporting limit at $0.032 \mu\text{g/L}$ in December 2008 to as high as $0.118 \mu\text{g/L}$ in September 2008. Primary and secondary treatment removed more than 78% of the metolachlor, which was detected just

above the reporting limit (0.025 µg/L) in effluent in September 2008. Metolachlor was not detectable in any of the RO permeate samples.

The regulatory levels for metolachlor in the ANZECC guidelines are 0.02 µg/L (low reliability) for freshwater ecosystems and 800 µg/L in water intended for recreational purposes. The *Australian guidelines for water recycling 2008 Phase 2* recommend a human health guideline of 300 µg/L for metolachlor. The September 2008 secondary effluent from Beenyup AWTP was slightly above the ANZECC guideline (0.025 µg/L versus a guideline of 0.02 µg/L) for freshwater ecosystems. The method reporting limit was also slightly higher than the guideline value, although it should be noted that due to the paucity of available toxicity data, the guideline is acknowledged as being of low reliability: it is several orders of magnitude lower than the health-based guideline proposed in the water recycling guidelines and the ANZECC guideline for recreational purposes.

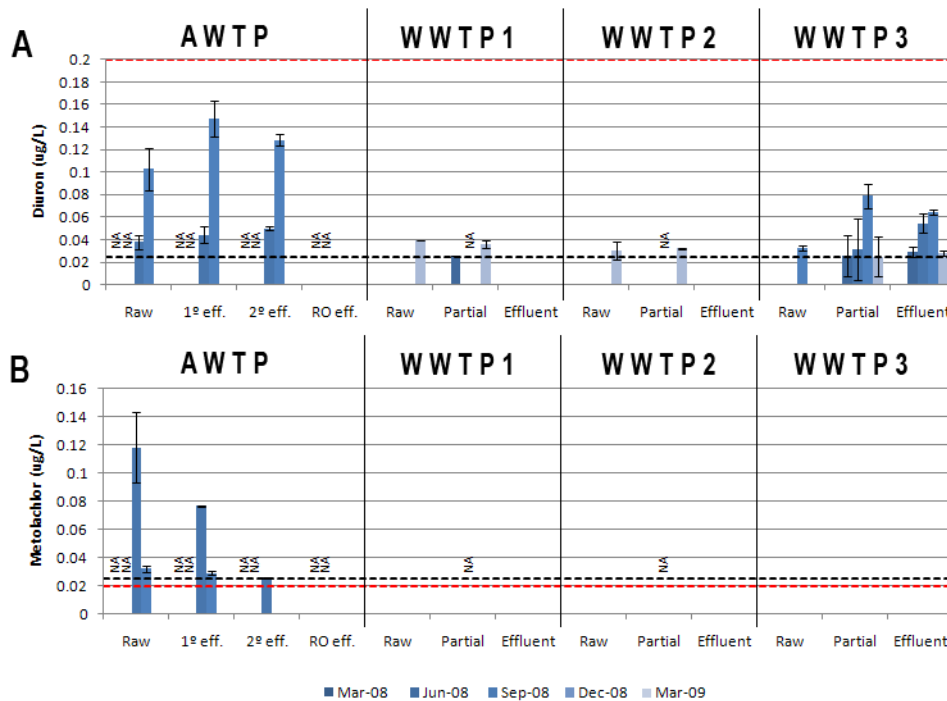


Figure 33: Concentration of diuron and metolachlor at the four plants

Notes: (A) Diuron and (B) metolachlor in samples from Beenyup (AWTP), Waroona (WWTP1), Harvey (WWTP2) and Gordon Road (WWTP3), in µg/L. The dashed black line represents the limit of reporting (0.025 µg/L). The dashed red line represents the ANZECC guidelines for fresh water (0.2 and 0.02 µg/L for diuron and metolachlor respectively). NA = not available.

3.2 Ecotoxicity toolbox bioanalysis

In general, the raw wastewater contained high levels of toxicity. Wastewater treatment effectively removed most of the toxicity, and the final effluents were below available guideline values. At Beenyup AWTP, the aerobic and anaerobic digestion and RO removed most of these toxic compounds, while the oxidation ditch at Gordon

Road WWTP generally performed well (although it seemed to create short-lived genotoxic compounds). The raw wastewater from Harvey and Waroona WWTPs was much less toxic than that of Beenyup AWTP and Gordon Road WWTP, and the local pond systems were adequate at reducing the toxicity to levels comparable with Beenyup secondary-treated wastewater and Gordon Road effluent. The site downstream of the Harvey WWTP discharge point exhibited slightly elevated toxicity compared with the upstream site, although it was always below available guidelines.

3.2.1 MicroLumo assay for baseline toxicity

The MicroLumo assay is a measure of acute cellular toxicity in bacteria. Overall, raw untreated wastewater was toxic to bacteria, but with wastewater treatment, this toxicity was greatly reduced. At all the treatment plants monitored in this study, the final effluent was well below the proposed USEPA guideline for bacterial toxicity.

At Beenyup AWTP, raw wastewater was very toxic to bacteria (Figure 34A), with an average activity above the 8.1 TUs of the USEPA guideline under consideration. This means that raw, untreated wastewater would negatively affect bacterial ecosystems. Primary sedimentation did not reduce the cytotoxicity, however aerobic and anaerobic digestion and clarification (secondary treatment) reduced baseline toxicity by more than 89% to less than 1 TU. This suggests that toxic chemicals are either mineralised to less toxic degradation products or absorbed by sludge particles and removed from the water stream. RO reduced cytotoxicity of the water stream by a further 68% at least, to below quantification limit (<0.15 TUs) in March and June 2008, 0.16 TUs in September 2008 and 0.41 TUs in December 2008 (Figure 34A). The toxicity of the final RO permeate was more than 95% lower than that of the raw influent. The low baseline toxicity present in December 2008 was almost 20 times less than the proposed guideline and only 2.7 times higher than that of the blank, suggesting that this residual toxicity is of no concern unless it is due to chemicals of a very specific potency (Escher 2008a). Specific toxicity was tested in the Max-I-PAM, E-SCREEN and AR-CALUX, none of which detected any activity in the RO permeate.

Baseline toxicity in raw influent from Gordon Road (WWTP3, Figure 34B) was similar in concentration to that of Beenyup AWTP, and sometimes higher than 8.1 TUs (September 2008). The oxidation ditch reduced bacterial cytotoxicity by 70 to 91%, with a further reduction of 18 to 39% from the clarification process. The final effluent from Gordon Road WWTP exhibited low baseline toxicity (average 0.76 TUs, or 10 times less than the proposed USEPA environmental guideline).

The two pond-based WWTPs – Waroona (WWTP1) and Harvey (WWTP2) – displayed similar trends (Figure 34B). Raw wastewater was not greatly cytotoxic (average of 2 TUs, or more than four times below the proposed guideline value of 8.1), and treatment in the ponds resulted in a 27 to 46% decrease in cytotoxicity. The additional ponds did not reduce baseline toxicity any further at Waroona WWTP, but resulted in an increase in baseline toxicity at Harvey WWTP in December 2008 and March 2009 (up to 6.08 and 2.73 TUs respectively; WWTP2 in Figure 34B). Baseline toxicity can be caused by many factors, and it only provides a very coarse measure of pollutant load (similar to a total organic carbon measurement). It is therefore

difficult to say what could have caused this increase, and the reactive (genotoxicity) and specific toxicity (photosynthesis inhibition, endocrine disrupting effects) must be carefully interpreted.

In the environmental samples, the sites immediately downstream of the Waroona and Harvey effluent discharge points (Dn1 and Dn2 in Figure 34C respectively) were 2.7 to 5.8 times more cytotoxic to bacteria than the corresponding upstream sites, but these concentrations were still far below the proposed environmental guideline of 8.1 TUs. The reference site sample (River, Figure 34C) also contained low levels of baseline toxicity (0.27 TUs, or 1.8 times higher than the blank). This is similar to the baseline toxicity detected in the RO permeate, and suggests that low activity is unlikely to be of concern, but rather is perhaps caused by non-specific toxicity due to enrichment of the water samples.

In previous Queensland studies using the MicroLumo assay, raw wastewater contained the equivalent of 1.2 TUs, secondary effluent up to 0.25 TUs, and final effluent up to 0.22 TUs (Muller 2006). This range is similar to the results presented in this report, with the exception being comparatively high cytotoxicity in Beenypup AWTP influent and primary treated samples, as well as Gordon Road WWTP influent.

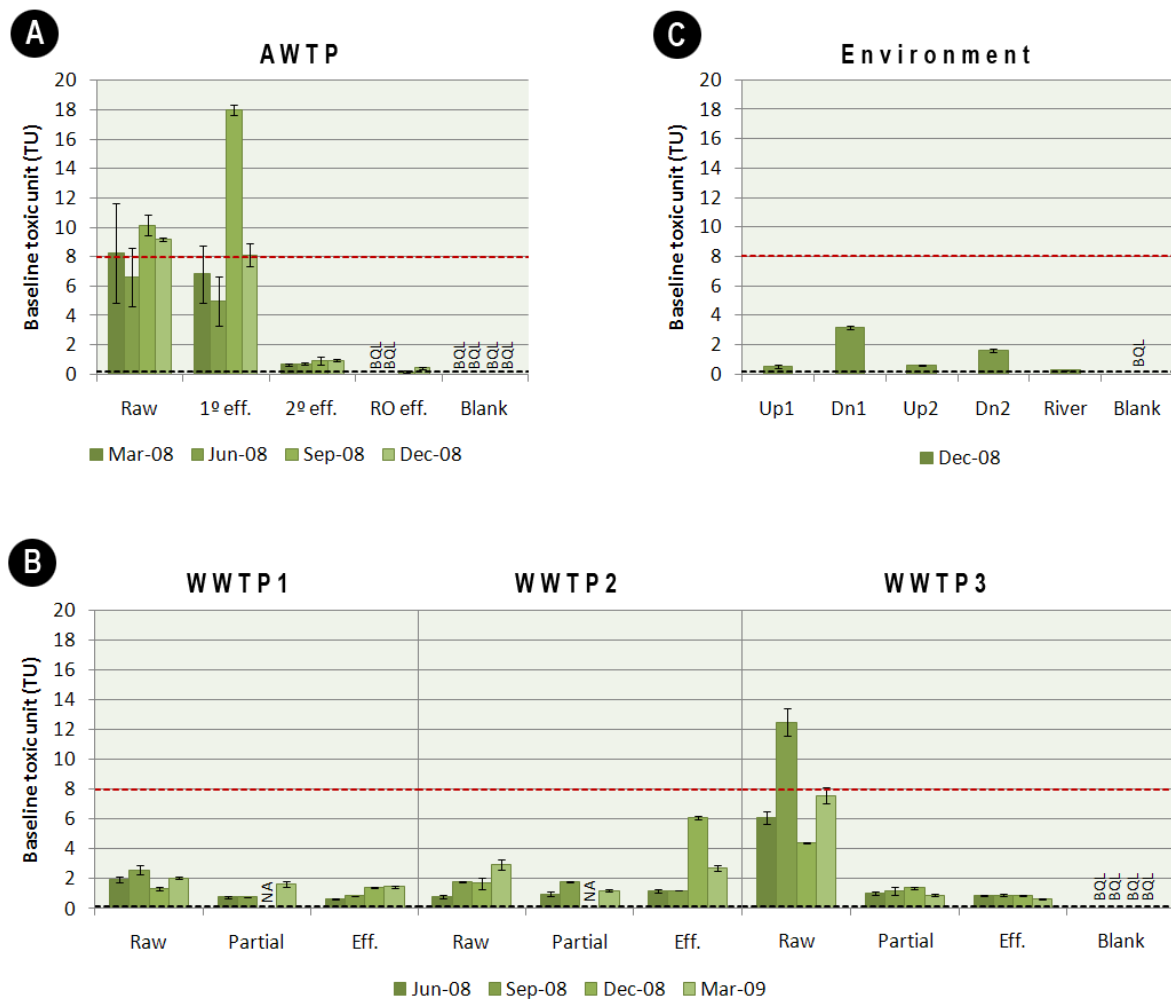


Figure 34: Cytotoxicity of the water samples assessed by MicroLumo assay

Notes: (A) Beenyup water reclamation plant (AWTP); (B) Waroona (WWTP1), Harvey (WWTP2) and Gordon Road (WWTP3) wastewater treatment plants; and (C) environmental samples upstream and downstream of Waroona (Up1 and Dn1 respectively), upstream and downstream of Harvey (Up2 and Dn2 respectively), and in the Serpentine River (River). Results are expressed as baseline toxicity unit (TU = 1/EC50 (mL)). The red dashed line indicates a suggested guideline based on proposed USEPA guidelines (equivalent to 8.1 TUs). The black dashed line indicates the method quantification limit (0.15 TUs). BQL = below quantification limit, NA = not available.

3.2.2 *umuC* assay for genotoxicity

The *umuC* assay measures the induction of the SOS response system, a biochemical DNA repair pathway induced in response to single-stranded DNA damage. It is a measure of the potential of a chemical to cause DNA damage (genotoxicity).

The assay is performed in two modes, with a liver enzyme fraction S9 (+S9) and without (-S9). The mode with liver enzymes (+S9) may be more representative of the

situation within the body of an exposed complex multi-cellular organism, while the mode without metabolism (-S9) is more representative of the situation for biologically-simple microorganisms. The two modes resulted in very similar trends, although the inclusion of the S9 fraction reduced the potential genotoxicity of all samples (figures 35 and 36). In other words some of the compounds responsible for genotoxicity were effectively detoxified by liver enzymes, suggesting a lower risk to exposed complex multi-cellular organisms.

At Beenyup AWTP, raw wastewater on average contained 1.34 and 0.34 TUs in the -S9 and +S9 fractions respectively (figures 35A and 36A). Primary sedimentation did not significantly reduce genotoxicity (average of 1.16 and 0.30 TUs for -S9 and +S9 respectively). Aerobic and anaerobic digestion resulted in a 15 to 44% decrease in genotoxicity, down to 0.74 and 0.29 TUs for -S9 and +S9 respectively. RO was the only really effective removal method for genotoxicity, removing at least 86% of the activity (to <0.04 TUs, the method quantification limit). Genotoxicity was not detected in any of the RO permeate samples (figures 35A and 36A).

Genotoxicity in raw untreated wastewater at Gordon Road WWTP was comparable with that of Beenyup AWTP, with an average of 2.69 and 0.52 TUs for -S9 and +S9 respectively (WWTP3, figures 35B and 36B). Oxidation resulted in a 4.9 to 6.2 fold increase in genotoxicity of the water stream. The solids have a residence time of up to four weeks in the oxidation ditch, and an increase in genotoxicity in such an environment favouring biochemical interactions is not unexpected. Importantly though, genotoxicity dropped significantly post-clarification (to 0.67 and 0.21 TUs for -S9 and +S9 respectively). The genotoxicity of the final effluent was 60 to 75% lower than that of the raw influent, and was similar to that of the secondary effluent at Beenyup AWTP.

Genotoxicity in raw wastewater at Waroona and Harvey WWTPs was lower than that of Beenyup AWTP and Gordon Road WWTP, with 0.10 to 0.56 and <0.04 to 0.22 TUs for -S9 and +S9 respectively (WWTP 1 and 2, figures 35B and 36B). The pond system did not greatly reduce genotoxicity, with the effluent genotoxicity ranging from <0.04 to 0.38 and <0.04 to 0.31 TUs for -S9 and +S9 respectively.

Genotoxicity was not detected in any of the river samples (figures 35C and 36C).

With the exception of samples from the oxidation ditch, all samples were below 5.5 GTUs, and all had to be concentrated to produce a genotoxic effect. Their discharge into the environment would therefore not be expected to produce significant genotoxicity. The samples from the oxidation ditch were, however, above 5.5 GTUs in the assay -S9, suggesting that if this water were discharged without further treatment (which is not the case) it might cause adverse effects in exposed microorganisms.

In previous studies, when the *umuC* assay has been used without metabolic activation (*umuC* -S9), wastewater from Queensland was found to have up to 2.1 TUs in raw wastewater and less than 0.06 TUs in treated effluent (Macova et al. in press). These numbers are similar to those presented in this report, with the exception of the high genotoxicity post-oxidation at Gordon Road WWTP.



Figure 35: Genotoxicity of the water samples assessed by *umuC* (+S9) bioassay

Notes: (A) Beenyup water reclamation plant (AWTP); B) Waroona (WWTP1), Harvey (WWTP2) and Gordon Road (WWTP3) wastewater treatment plants; and C) environmental samples upstream and downstream of Waroona (Up1 and Dn1 respectively), upstream and downstream of Harvey (Up2 and Dn2 respectively), and in the Serpentine River (River). Results as genotoxic unit (GTU = 1 / equivalent volume for IR 1.5 (mL)). The black dashed line represents the method quantification limit at 0.04 TUs. The red dashed line represents significant genotoxicity, at 5.5 GTUs. BQL = below quantification limit, NA = not available

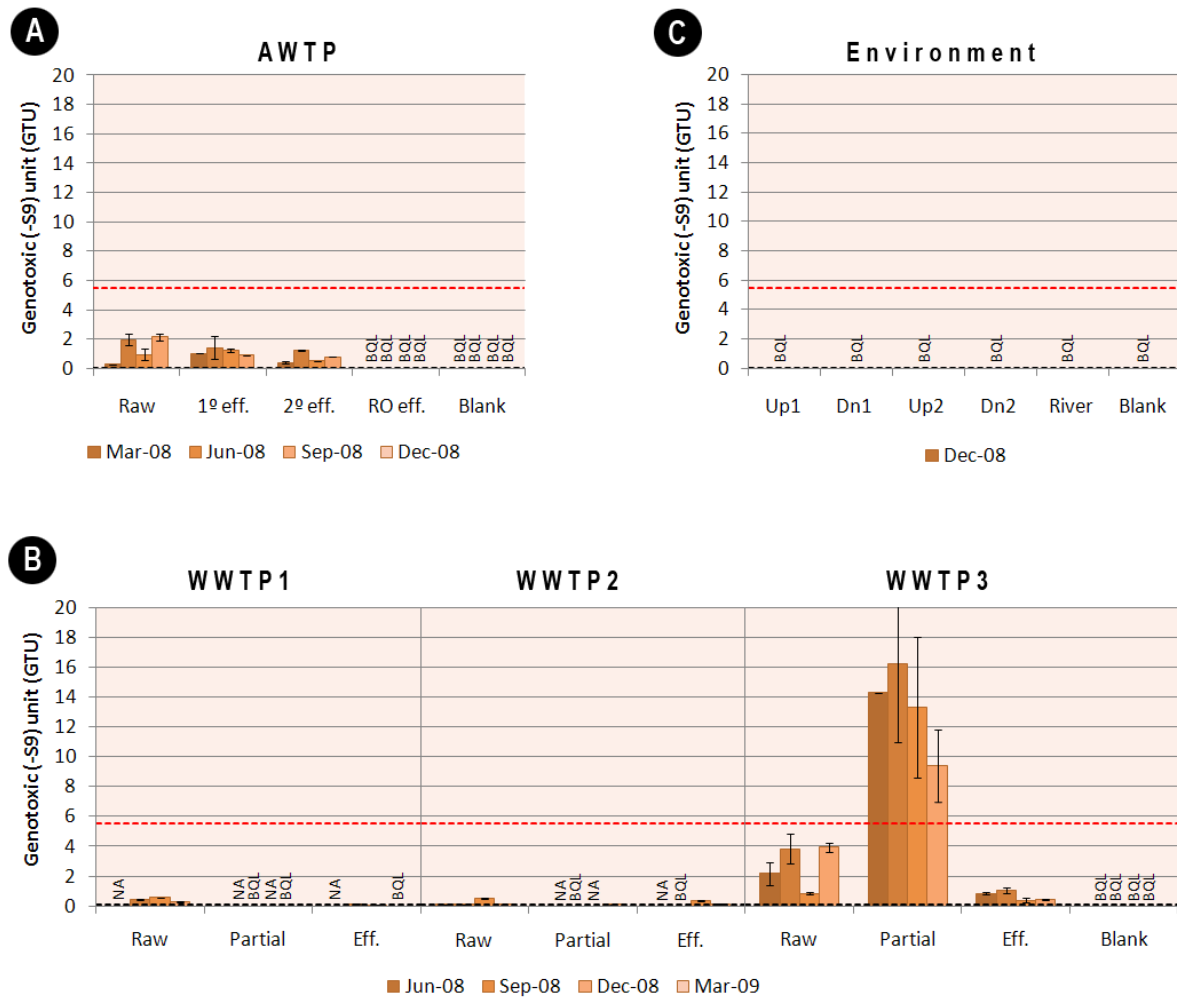


Figure 36: Genotoxicity of the water samples assessed by *umuC* (-S9) bioassay

Notes: (A) Beenyup water reclamation plant (AWTP); (B) Waroona (WWTP1), Harvey (WWTP2) and Gordon Road (WWTP3) wastewater treatment plants; and (C) environmental samples upstream and downstream of Waroona (Up1 and Dn1 respectively), upstream and downstream of Harvey (Up2 and Dn2 respectively), and in the Serpentine River (River). Results as genotoxic unit (GTU = 1 / equivalent volume for IR 1.5 (mL)). The black dashed line represents the method quantification limit at 0.04 TUs. The red dashed line represents significant genotoxicity, at 5.5 GTUs. BQL = below quantification limit, NA = not available.

3.2.3 Max-I-PAM assay for photosynthesis inhibition

The Max-I-PAM assay measures inhibition of photosynthesis in organisms such as plants, algae and bacteria. This endpoint is particularly sensitive to herbicides.

Raw wastewater from Beenyup AWTP strongly inhibited photosynthesis, equivalent to 0.40 $\mu\text{g/L}$ of diuron on average (Figure 37A). Primary sedimentation had little effect, but aerobic and anaerobic digestion reduced the phytotoxic activity by 48 to 90%, down to 0.08 $\mu\text{g/L}$ DEq (below the ANZECC/ARMCANZ freshwater guideline of

0.2 µg/L). RO removed a further 48% at least, and all RO permeate samples were below the quantification limit (<0.03 µg/L DEq; Figure 37A).

Raw wastewater from Gordon Road WWTP was similar to that of Beenyup AWTP (with 0.30 µg/L DEq on average; WWTP3 in Figure 37B). The oxidation ditch generally reduced this by 58 to 83% (except in December 2008, when it caused a slight increase). The phytotoxic activity in the final effluent was 50 to 87% lower than that of raw wastewater, at 0.04 to 0.07 µg/L DEq, below the ANZECC/ARMCANZ (2000) freshwater guideline of 0.2 µg/L for diuron.

The phytotoxic activity in raw wastewater from Waroona and Harvey WWTPs was similar, ranging from 0.04 to 0.23 µg/L DEq (WWTP 1 and 2 respectively; Figure 37B). The overall removal efficacy with the pond-based systems was 47 to 59% on average. The phytotoxic activity in the final effluent was similar to that of Beenyup AWTP's secondary-treated effluent and Gordon Road WWTP's treated effluent, ranging from <0.03 to 0.08 µg/L DEq.

With the river water samples, photosynthesis inhibition was only detected downstream of the Harvey WWTP discharge point (Dn2, Figure 37C).

Recent studies using the Max-I-PAM in Queensland reported up to 0.22 µg/L DEq in raw wastewater and up to 0.05 µg/L DEq in treated wastewater (Macova et al. in press; Reungoat et al. in press). Similar concentrations have been reported in Switzerland, with an average of 0.28 µg/L DEq in raw wastewater, 0.19 µg/L DEq in treated effluent, and up to 0.23 µg/L DEq in river water. Up to 1 µg/L DEq has been reported in primary effluent in Switzerland (Escher et al. 2009). Another Queensland study reported up to 0.04 µg/L in river water (Escher et al. 2006). The concentrations for Western Australia presented in this study are very similar to those reported in Queensland and are lower than those reported in Switzerland.

The ANZECC/ARMCANZ (2000) guideline for water intended for irrigation is 2 µg/L for diuron. The levels detected in all plant effluents in this study were at least an order of magnitude lower than this guideline value (<0.2 µg/L), suggesting that as far as herbicides are concerned, the effluent produced by these WWTPs is of adequate quality for irrigation.

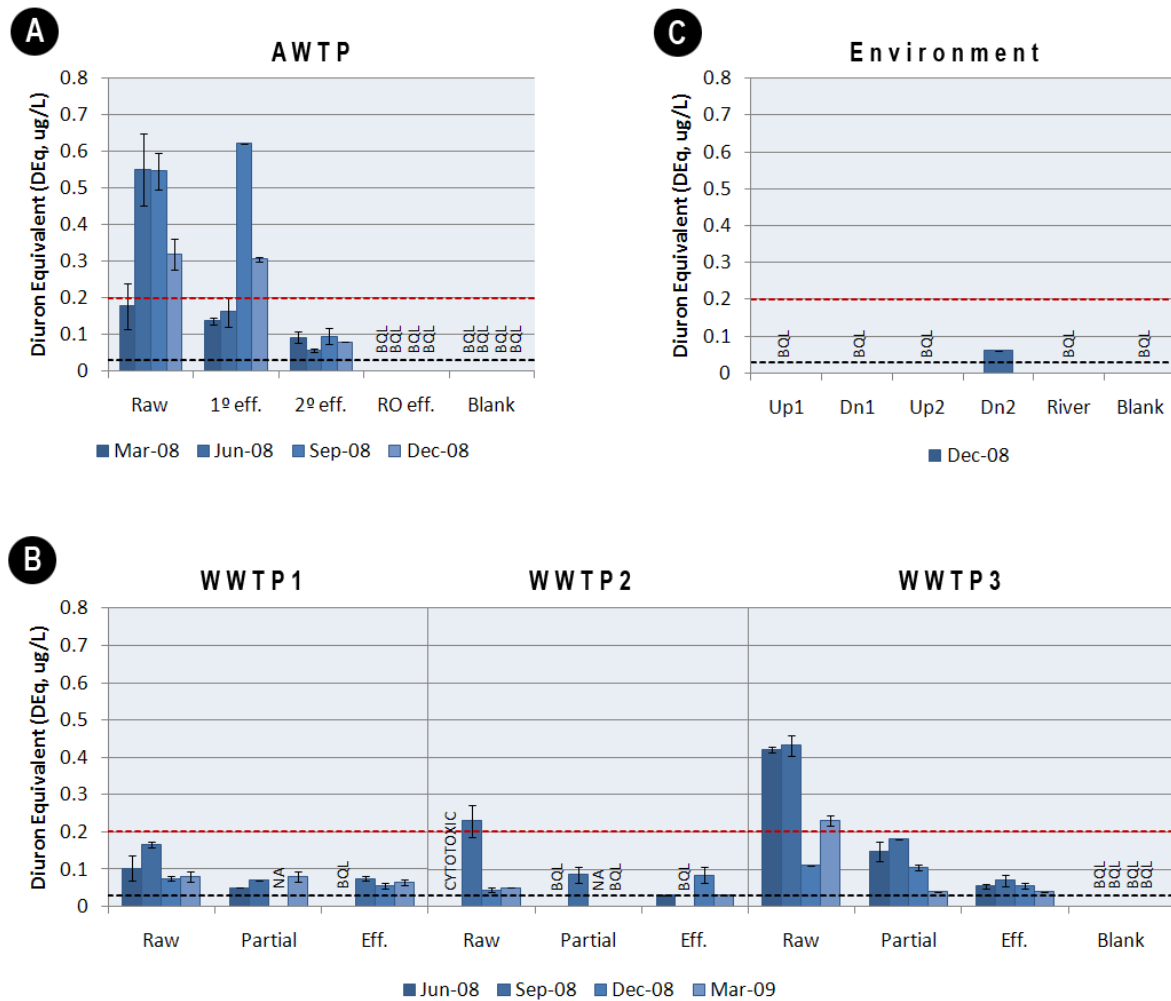


Figure 37: Photosynthesis inhibition of the water samples assessed by Max-I-PAM bioassay

Notes: (A) Beenyup water reclamation plant (AWTP); (B) Waroona (WWTP1), Harvey (WWTP2) and Gordon Road (WWTP3) wastewater treatment plants; and (C) environmental samples upstream and downstream of Waroona (Up1 and Dn1 respectively), upstream and downstream of Harvey (Up2 and Dn2 respectively), and in the Serpentine River (River). Results are expressed as diuron equivalent (DEq, µg/L). The red dashed line indicates a suggested guideline based ANZECC guidelines for fresh water (0.2 µg/L). The black dashed line indicates the method quantification limit (0.03 µg/L DEq). BQL = below quantification limit, NA = not available.

3.2.4 E-SCREEN assay for estrogenic EDCs

The E-SCREEN assay measures estrogenic activity, which at high concentrations could cause feminisation in exposed organisms (a form of endocrine disruption). Some known estrogenic EDCs include estrogen hormones (estradiol, estrone, estriol), pharmaceutical estrogens (mestranol, ethynylestradiol), and industrial estrogen mimics (such as bisphenol A, nonylphenol and phthalates).

Raw wastewater from Beenyup AWTP contained high levels of estrogenic compounds, from 36.6 to 101 ng/L 17β-estradiol equivalents (EEq) (Figure 38A). In

three out of four cases, this activity was increased during primary treatment, a well-recognised phenomenon thought to be due to the reactivation of conjugated hormones or conversion into more potent metabolites (Leusch 2005a). The combination of primary sedimentation and aerobic and anaerobic digestion, however, resulted in a significant decrease in estrogenicity of 82 to 98%. This is consistent with predictions that estrogens will mineralise and be adsorbed by sludge effectively (Andersen et al. 2003). Estrogenicity in the secondary-treated effluents ranged from 1.64 to 6.64 ng/L EEq. RO removed at least 39% of the remaining estrogenicity, and all RO permeate samples were below the quantification limit of the assay (<1 ng/L EEq).

Estrogenic activity in raw wastewater at Gordon Road WWTP was slightly lower than that of Beenyup AWTP, ranging from 14.8 to 54.3 ng/L EEq (WWTP3, Figure 38B). The oxidation ditch caused an increase in estrogenic activity in half the samples and a decrease in the other half. Besides June 2008, estrogenic activity in the final effluent was greatly reduced to a level similar to that of secondary-treated wastewater from Beenyup AWTP, ranging from <1 to 4.39 ng/L EEq (WWTP3, Figure 38B). In June 2008, however, the activity of the final effluent was 21.6 ng/L EEq, a concentration that would likely cause endocrine-disrupting impacts in the receiving ecosystem if maintained for a longer period of time. At the subsequent sampling event (September 2008), the estrogenic activity of the final effluent had dropped significantly (<1 ng/L EEq) (WWTP3, Figure 38B).

Estrogenic activity in raw wastewater at both Waroona and Harvey WWTPs was very low (less than 4.8 ng/L EEq), and below the quantification limit at all other treatment stages (WWTP1 and WWTP2 in Figure 38B respectively).

The river samples generally did not display any estrogenic activity with the exception of the site immediately downstream of Harvey WWTP (Dn2, Figure 38C).

The *Australian guideline for water recycling 2008 Phase 2* (NRMMC/EPHC/NHMRC 2008) proposes a guideline of 275 ng/L for 17 β -estradiol in water intended for potable reuse. The concentrations measured in this study were significantly lower than that of the guideline value. There is no accepted environmental guideline, but it is widely accepted that estrogenic effects can occur in fish exposed to concentrations as low as 1 ng/L 17 β -estradiol (Routledge et al. 1998; Metcalfe et al. 2001; Thorpe et al. 2003; Kidd et al. 2007). The secondary-treated wastewater from Beenyup AWTP and the final effluent from Gordon Road WWTP are slightly above this concentration, and more work is needed to determine if these discharges are having any impact on the receiving ecosystems.

These concentrations are within the expected range based on previous monitoring work (Tan et al. 2007a; GWRC 2008; LWA 2007). Recent studies using the E-SCREEN in Queensland have reported up to 74 ng/L EEq in raw wastewater, up to 1.6 ng/L EEq in secondary-treated wastewater, and less than 0.06 ng/L EEq in highly-treated effluent (GWRC 2008; Macova et al. in press; Reungoat et al. in press). Estrogenic activity in river water in Queensland has been reported as high as 0.47 ng/L (GWRC 2008).

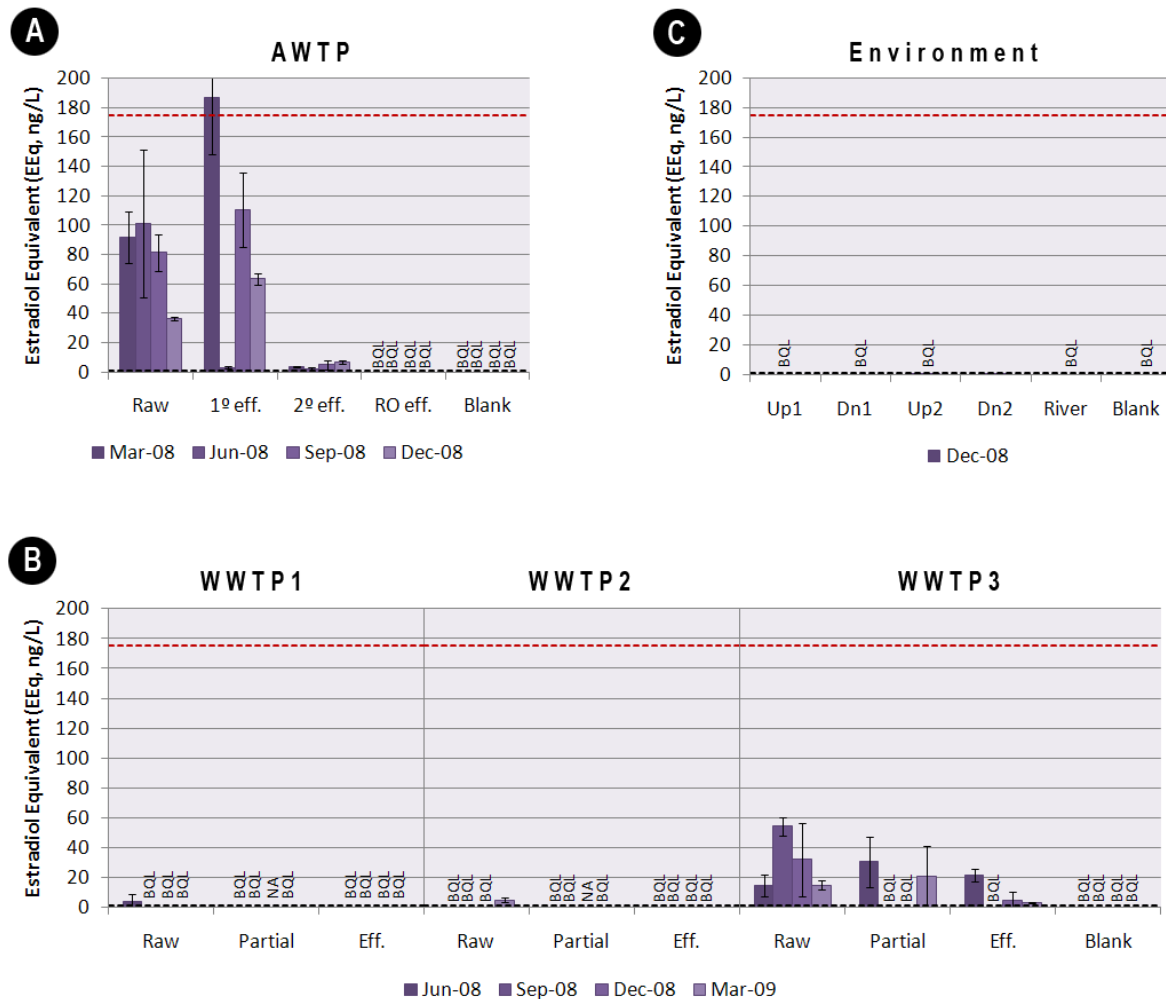


Figure 38: Estrogenicity of the water samples assessed by E-SCREEN bioassay

Notes: (A) Beenyup water reclamation plant (AWTP); (B) Waroona (WWTP1), Harvey (WWTP2) and Gordon Road (WWTP3) wastewater treatment plants; and (C) environmental samples upstream and downstream of Waroona (Up1 and Dn1 respectively), upstream and downstream of Harvey (Up2 and Dn2 respectively), and in the Serpentine River (River). Results are expressed as estradiol equivalent (EEq, ng/L). The red dashed line indicates a suggested guideline based on the *Australian guidelines for water recycling 2008 Phase 2* (175 ng/L for 17 β -estradiol). The black dashed line indicates the method quantification limit (1 ng/L EEq). BQL = below quantification limit, NA = not available.

3.2.5 AR-CALUX assay for androgenic EDCs

The AR-CALUX measures the activity of androgenic chemicals, which at high concentrations could cause masculinisation of exposed organisms (a form of endocrine disruption).

Raw wastewater at Beenyup AWTP again contained high levels of androgenic compounds, from 22.9 to 87.3 ng/L dihydrotestosterone equivalents (DHT Eq) (Figure 39A). Similar to estrogenicity, the androgenic activity was increased during

primary treatment, possibly due to reactivation of conjugated hormones or degradation into a more potent androgen. Secondary treatment was, however, very effective at removing that activity to below the quantification limit of the assay (<2.5 ng/L), a removal efficacy of at least 92%. All RO permeate samples were also below the quantification limit of the assay.

The androgenic activity in raw wastewater at Gordon Road WWTP was slightly lower than that of Beenyup AWTP, ranging from 12.3 to 22.9 ng/L DHT Eq (WWTP3, Figure 39B). The activity was efficiently removed to below the quantification limit (<2.5 ng/L) by the oxidation ditch (a removal of at least 79.7%). There was no detectable androgenic activity in the final effluent.

As was the case with estrogenic activity, androgenic activity at Waroona and Harvey WWTPs was very low and only detectable in raw wastewater (ranging from <2.5 to 16.1 ng/L DHT Eq) (WWTP1 and WWTP2 respectively, Figure 39B).

None of the environmental (drain) samples had any detectable level of androgenic activity (all samples <2.5 ng/L DHT Eq) (Figure 39C).

There is no guideline for DHT in Australia, however the guideline of 7 µg/L for testosterone in the *Australian guidelines for water recycling 2008 Phase 2* (NRMMC/EPHC/NHMRC 2008) can be translated to a DHT equivalent guideline of 1500 ng/L. The concentrations measured in this study were all at least one to two orders of magnitude lower than the guideline (even in raw wastewater), suggesting a low risk to human health from these waters. There is no accepted environmental guideline for androgens, and little is available in the literature. In a 2002 study, 3 µg/L of dihydrotestosterone was required to significantly induce the production of an androgen-dependent protein in fish (Katsiadaki et al. 2002). All effluents tested in this study were below the quantification limit of the AR-CALUX assay (<2.5 ng/L) and three orders of magnitude lower than that used by the 2002 study. Androgenic endocrine disruption is therefore unlikely in the receiving environments.

Few studies have monitored androgenic activity. A study in south-east Queensland previously reported high androgenicity in wastewater, ranging from 2000 to 9000 ng/L testosterone equivalent (TEq) in raw wastewater and from <6.5 up to 600 ng/L TEq in treated wastewater (Leusch et al. 2006b). That study used a receptor-binding bioassay, which is a measure of androgenicity at the molecular level, and can result in higher quantification than cellular methods such as the AR-CALUX (Leusch et al. 2005a). A study in The Netherlands using the AR-CALUX showed concentrations of androgenic activity in raw wastewater (81 to 86 ng/L DHT Eq) similar to those reported here and very low concentrations in treated wastewater (0.75 to 0.83 ng/L DHT Eq) (van der Linden et al. 2008).

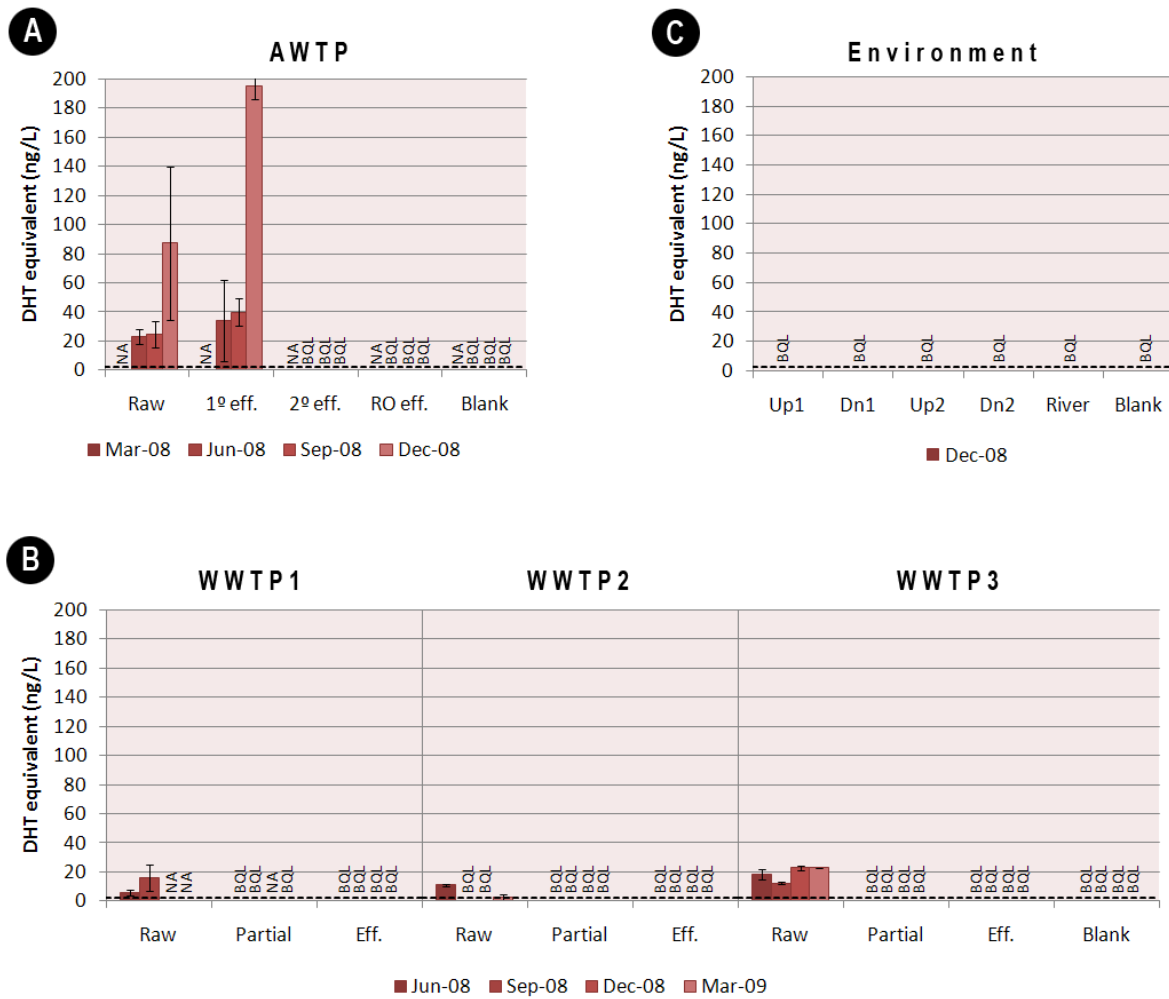


Figure 39: Androgenicity of the water samples assessed by AR-CALUX bioassay

Notes: (A) Beenyup water reclamation plant (AWTP); (B) Waroona (WWTP1), Harvey (WWTP2) and Gordon Road (WWTP3) wastewater treatment plants; and (C) environmental samples upstream and downstream of Waroona (Up1 and Dn1 respectively), upstream and downstream of Harvey (Up2 and Dn2 respectively), and in the Serpentine River (River). Results are expressed as dihydrotestosterone equivalent (DHT Eq, ng/L). The red dashed line indicates a suggested guideline based on the *Australian guidelines for water recycling 2008 Phase 2* (7000 ng/L for testosterone, translated to a DHT Eq of 1500 ng/L). The black dashed line indicates the method quantification limit (2.5 ng/L DHT Eq). BQL = below quantification limit, NA = not available.

3.3 In situ measurements

3.3.1 Physical and chemical parameters

The water temperature at all three locations was lower during the September and July (winter) sampling periods than the December and March (summer) sampling periods, but were similar between upstream and downstream sampling sites of both WWTPs. The water at the reference site at Serpentine River was cooler in winter than the WWTP sites, but summer temperatures were similar. Dissolved oxygen (DO) concentrations varied between sampling periods at all sites. DO in winter was

lower than in summer but did not decrease to levels expected to affect fish health. The pH did not vary greatly across sampling times except for an increase at sites upstream and downstream of the Harvey WWTP in the July 2009 sampling period. Similarly, the salinity at the sites was generally consistent across sampling periods with the result at Serpentine National Park lower than the other locations.

Table 7: Site locations and water physical and chemical parameters of the study sites at the four sampling times at Harvey WWTP

Harvey	Upstream				Downstream			
	S:330 05' 27.19" E:1150 53' 13.41"				S: 330 05' 25.19" E: 1150 53' 34.42"			
	Sep-08	Dec-08	Mar-09	Jul-09	Sep-08	Dec-08	Mar-09	Jul-09
Temp (°C)	15.2	24.8	22.6	16.0	16.8	24.2	23.2	15.7
DO (mg/L)	10.15	6.75	8.46	12.95	10.93	4.19	3.20	8.46
pH	7.05	7.05	7.14	9.80	7.30	6.85	6.82	10.48
Sal (mg/L)	246	180	160	160	273	210	200	180

Table 8: Site locations and water physical and chemical parameters of the study sites at the four sampling times at Waroona WWTP

Waroona	Upstream				Downstream			
	S:320 50' 21.82" E:1150 54' 47.03"				S: 320 50' 10.33" E: 1150 54' 47.03"			
	Sep-08	Dec-08	Mar-09	Jul-09	Sep-08	Dec-08	Mar-09	Jul-09
Temp (°C)	17.1	n.d.	20.1	14.0	14.8	n.d.	20.0	13.3
DO (mg/L)	10.50	n.d.	9.31	11.84	12.20	n.d.	7.10	11.60
pH	7.32	n.d.	7.10	8.20	7.45	n.d.	6.93	7.40
Sal (ppm)	236	n.d.	110	200	221	n.d.	240	190

Table 9: Site locations and water physical and chemical parameters of the study sites at the four sampling times at Serpentine National Park reference site

	S: 320 22' 07.47" E:1160 00' 06.42"			
	Sep-08	Dec-08	Mar-09	Jul-09
Temp (°C)	14.2	22.4	21.6	11.7
DO (mg/L)	12.23	6.70	6.02	11.60
pH	7.23	7.42	6.48	6.59
Sal (ppm)	155	160	120	160

The temperature at all sites followed a typical temperature pattern of warming during summer and cooling through to winter. The fact that Serpentine National Park had cooler water temperatures in winter was expected – given this stream is highly shaded in most of the upstream reaches. While little difference in the DO content occurred between upstream and downstream WWTP sites at individual sampling times, there was a consistent decrease during summer. This corresponds with the known inverse relationship between DO and water temperature but is also likely to be associated with the significantly lower water levels observed at all sites during summer. In general, the sites were similar in most variables and therefore comparisons between the fish populations inhabiting them were considered appropriate.

Mosquitofish are tolerant of a wide range of environmental parameters. The observed physical and chemical variables were within the known acceptable range for this species (Pyke 2006).

3.3.2 Population demography

A total of 1832 mosquitofish were captured during the study (Table 10). The catch rate was generally higher at the two Harvey WWTP sites than at either the Waroona WWTP or Serpentine sites. This was particularly so in July 2009 when catch rates at Waroona and Serpentine were very low (< 50 fish per site). At all sites, and at all sampling times, adult male (fish with a fully developed gonopodium), juvenile male (fish with gonopodial development but no terminal complex of hooks), adult female and juvenile female fish were captured.

3.3.3 Sex ratio

There were significant differences between sex ratios upstream and downstream of both WWTPs in the September and March sampling periods, but not during the July sampling period (Figure 40). The trends were not consistent between sampling periods, with (statistically) significantly fewer males at the Harvey WWTP downstream site than the upstream site in September ($p = 0.002$), but significantly more males downstream than upstream in March ($p = 0.005$). The opposite trend was observed at the Waroona WWTP, where there were significantly fewer males at the downstream site than the upstream site in March ($p < 0.001$) and significantly more males at the upstream site in September ($p = 0.005$).

Table 10: Sex and developmental stage of fish captured during the study

		Harvey				Waroona				Serpentine	
		Upstream		Downstream		Upstream		Downstream		Total	PoP
Sep-08	Total		PoP	Total	PoP	Total	PoP	Total	PoP	Total	PoP
	Undifferentiated	15	0.04	0	0.00	0	0.00	0	0.00	5	0.06
Male	Adult	84 (0.48)	0.23	11 (0.34)	0.12	28 (0.42)	0.14	14 (0.34)	0.16	6 (0.21)	0.08
	Juvenile	90 (0.52)	0.25	21 (0.66)	0.22	39 (0.58)	0.20	25 (0.61)	0.29	22 (0.79)	0.28
	Total	174	0.48	32	0.34	67	0.34	41	0.48	28	0.36
Female	Adult	42 (0.24)	0.12	41 (0.65)	0.53	82 (0.63)	0.42	18 (0.41)	0.21	29 (0.64)	0.37
	Juvenile	134 (0.76)	0.37	22 (0.35)	0.64	48 (0.37)	0.24	26 (0.59)	0.31	16 (0.36)	0.21
	Total	176	0.48	63	0.66	130	0.66	44	0.52	45	0.58
Total	Total	365		95		197		85		78	
Mar-09	Undifferentiated	7	0.05	9	0.07	17	0.12	5	0.05	4	0.03
Male	Adult	40 (0.58)	0.31	37 (0.46)	0.29	48 (0.56)	0.34	16 (0.48)	0.16	47 (0.61)	0.34
	Juvenile	29 (0.42)	0.22	43 (0.54)	0.34	37 (0.44)	0.26	16 (0.48)	0.16	30 (0.39)	0.22
	Total	69	0.53	80	0.63	85	0.59	33	0.34	77	0.56
Female	Adult	29 (0.53)	0.22	26 (0.68)	0.20	31 (0.76)	0.22	47 (0.78)	0.48	37 (0.65)	0.27
	Juvenile	26 (0.47)	0.20	12 (0.32)	0.09	10 (0.24)	0.07	13 (0.22)	0.13	20 (0.35)	0.14
	Total	55	0.42	38	0.30	41	0.29	60	0.61	57	0.41
Total	Total	131		127		143		98		138	
Jun-09	Undifferentiated	11	0.05	3	0.04	0	0.00	3	0.13	0	0.00
Male	Adult	50 (0.43)	0.23	14 (0.37)	0.19	6 (0.86)	0.38	5 (0.71)	0.22	9 (0.50)	0.21
	Juvenile	67 (0.57)	0.30	24 (0.63)	0.33	1 (0.14)	0.06	2 (0.29)	0.09	9 (0.50)	0.21
	Total	117	0.53	38	0.53	7	0.44	7	0.30	18	0.43
Female	Adult	15 (0.16)	0.07	9 (0.29)	0.13	7 (0.78)	0.44	9 (0.69)	0.39	17 (0.71)	0.40
	Juvenile	79 (0.84)	0.36	22 (0.71)	0.31	2 (0.22)	0.13	4 (0.31)	0.17	8 (0.32)	0.19
	Total	94	0.42	31	0.43	9	0.56	13	0.57	24	0.57
Total	Total	222		72		16		23		42	

Figures in brackets are the proportion the developmental stage contributed to the total number of fish captured of that sex. PoP = proportional contribution to the population.

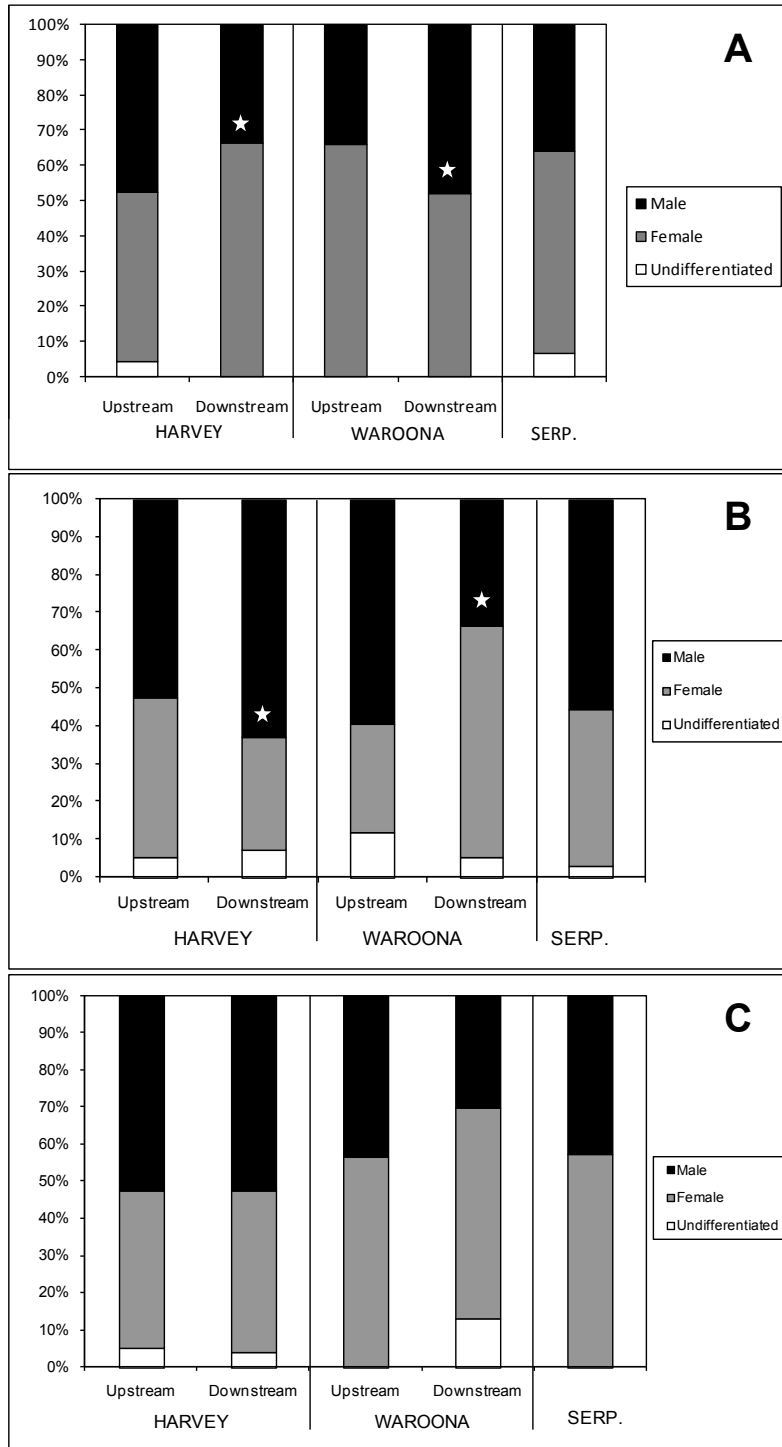


Figure 40: Percent sex ratio at the five collection sites

Notes: (A) September 2008; (B) March 2009; (C) July 2009. Male and female differentiation as described in section 2.4.2. The symbol * denotes significant difference to the relevant upstream site.

3.3.4 Morphological measurements

To separate juvenile male fish from males whose development had been potentially delayed by exposure to EDCs, 99% confidence intervals were drawn about the

centroid of adult male standard length and gonopodium length (Figure 41). Only fish falling within this range were included in the mosquitofish morphology investigation.

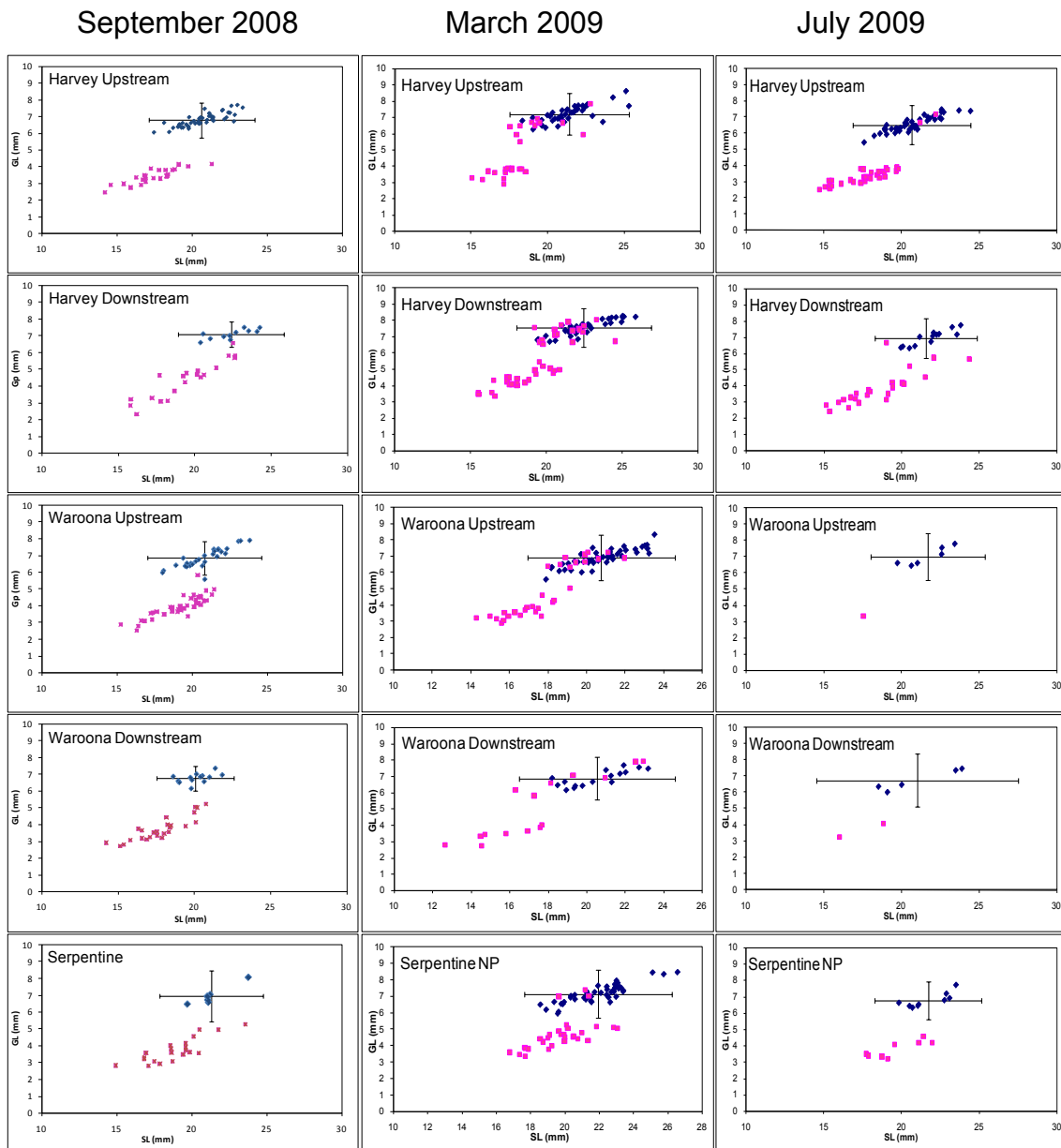


Figure 41: Relationship between the standard length and gonopodial length as an estimate of male sexual development at the five sampling sites

Notes: September 2008 (column 1), March 2009 (column 2) and July 2009 (column 3). Blue markers are male fish with fully developed gonopodia (with a terminal complex of hooks). Pink markers are male fish with gonopodial development but no terminal complex of hooks. Crosshairs represent 99% confidence intervals around the centroid of adult fish. This measure was used as a threshold for inclusion in gonopodial analyses.

There was no significant difference between the upstream and downstream sites at Harvey WWTP in terms of GL (ANCOVA, $p = 0.112$) or PAL (ANCOVA, $p = 0.104$) of

fish captured in September 2008 (Figure 42). Similarly, there was no significant difference between either the GL (ANCOVA, $p = 0.214$) or PAL (ANCOVA, $p = 0.546$) of fish captured upstream and downstream of Waroona WWTP in September 2008 (Figure 42). In March there was no significant difference between the sites upstream and downstream at Harvey WWTP in terms of GL (ANCOVA, $p = 0.323$) or PAL (ANCOVA, $p = 0.324$) (Figure 43). However, when compared with fish from the reference site at Serpentine National Park, fish from the downstream Harvey WWTP site had longer gonopodia (ANCOVA, $p = 0.001$) and longer PAL (ANCOVA, $p < 0.001$) (Figure 43). Fish from upstream and downstream of Waroona WWTP had similar GLs (ANCOVA, $p = 0.083$) but fish from downstream of the WWTP had shorter PALs (ANCOVA, $p = 0.038$) in the March sampling period (Figure 43). There was no difference between the GL (ANCOVA, $p = 0.472$) or PAL (ANCOVA, $p = 0.091$) at any of the sites sampled during July 2009 (Figure 44).

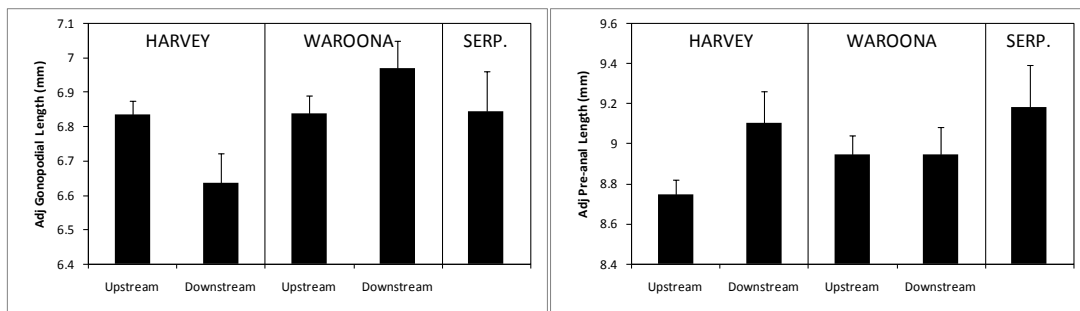


Figure 42: Gonopodial length (GL) and pre-anal length (PAL) of adult male mosquitofish from southern WWTP sampling sites in September 2008

Notes: Values are mean \pm standard error, adjusted during statistical analysis against a covariate (standard length) mean of 20.84 mm.

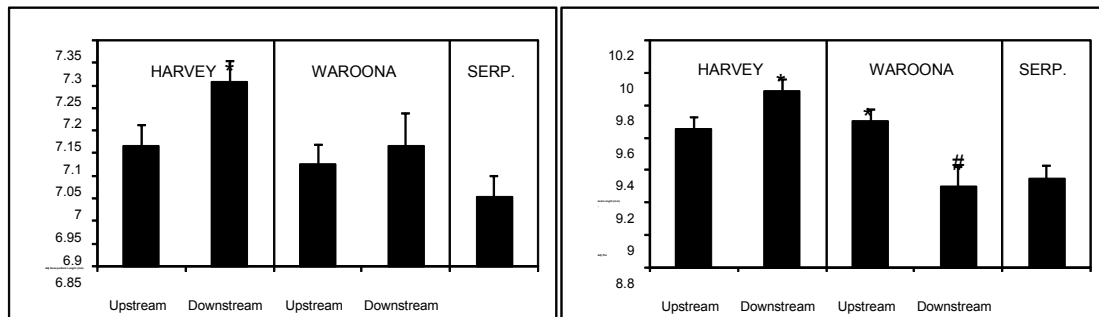


Figure 43: Gonopodial length (GL) and pre-anal length (PAL) of adult male mosquitofish from southern WWTP sampling sites in March 2009

Notes: Values are mean \pm standard error, adjusted during statistical analysis against a covariate (standard length) mean of 21.29 mm. The symbol * denotes significant difference between the site and the Serpentine National Park. The symbol # represents significant difference between upstream and downstream sites.

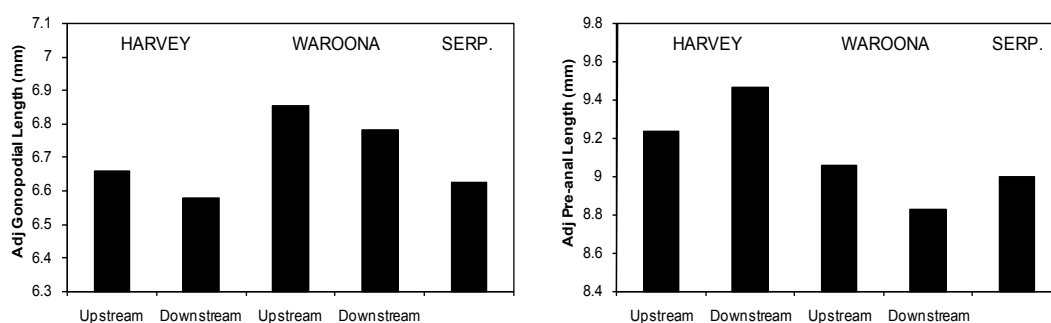


Figure 44: Gonopodial length (GL) and pre-anal length (PAL) of adult male mosquitofish from southern WWTP sampling sites in July 2009

Notes: Values are mean \pm standard error, adjusted during statistical analysis against a covariate (standard length) mean of 21.13 mm.

Male secondary sexual characteristics of mosquitofish have been widely used in Australia and overseas in both laboratory and field assessments of exposure to natural and synthetic steroid hormones (Bortone & Cody 1999; Dreze et al. 2000; Howell et al. 1980). In Australia, reductions in gonopodium length and a lack of anterior translocation of the gonopodium are the most common endpoints used in such assessments: they have been well validated in laboratory exposures identifying broad estimates of the lowest observable effects concentration (LOEC) and no observable effects concentration (NOEC) (Doyle et al. 2003; Doyle & Lim 2002). Such laboratory studies have also identified that certain life-stages are more sensitive to EDC exposure, during which such exposure will have lasting effects (Rawson et al. 2006). In field studies, the estrogenic impacts of WWTP effluent on the reproductive morphology of male mosquitofish have been measured in eastern Australia (Batty & Lim 1999; Leusch et al. 2006a; Rawson et al. 2008) and overseas (Angus et al. 2002).

This study examined the impact of treated wastewater discharge to drainage channels on resident populations of mosquitofish by examining the GL and PAL of male fish across three sampling periods. The results varied between the sampling periods but indicated no consistent estrogenic or androgenic impact of either the Harvey or Waroona WWTP effluents. In previous studies, significant differences in reproductive morphology have been noted over short geographic ranges with input of WWTP effluent (Batty & Lim 1999; Rawson et al. 2008). In this case the sites were separated by sufficient distance to ensure no migration of mosquitofish between upstream and downstream sites. Therefore any estrogenic impact of the WWTP effluent would have resulted in a noted decrease in gonopodial length and an increase in pre-anal length, neither of which was observed here.

The only significant difference observed between the upstream and downstream sites was at Waroona in summer (March 2009), when male fish had significantly shorter PALs downstream of the WWTP compared with fish at the upstream site.

While this potentially represents an androgenic effect, it was not a response repeated at any of the other endpoints for any other sampling period. While exposure to androgenic compounds has been shown to cause precocious gonopodial development and a shortening of the PAL, there is no empirical evidence that exposure to an androgen will decrease the PAL over the full developmental cycle. During normal male development, somatic growth ceases when gonopodial maturation is attained, but it is unclear whether this is the case where precocious maturation has occurred. If so, treating this metric statistically, taking the size of the fish into account will result in a correct interpretation of an androgenic effect, however – in the absence of the empirical evidence described above – the result observed here is tentative.

In addition to comparisons between upstream and downstream sites, comparisons were made to the reference site, Serpentine National Park, which does not receive WWTP effluent. Of particular interest are the comparisons between the sites upstream of both Harvey and Waroona WWTPs and this site – which ensure its validity as a reference site. For each sampling period there was no difference between the gonopodial length of fish upstream of either WWTP and those from Serpentine National Park. Only during summer at Waroona was there a difference between the PAL of fish upstream of the WWTP and Serpentine (this was an increase denoting an estrogenic effect). As such, we can conclude that while physical differences occurred between the Serpentine site and the WWTP upstream sites (temperature in particular), in terms of gonopodial morphology the choice of reference site was valid. The fact that no observable differences between the reference site and the upstream sites were found suggests no ongoing effect on fish reproductive morphology due to diffuse sources of EDCs through the highly agricultural catchments.

In the use of mosquitofish gonopodial morphology for assessment of EDC impacts, an issue that arises regularly is which animals to include and which to exclude from statistical analyses. Ideally all adult fish should be included in the analysis and juvenile fish excluded. However, this problem has its basis in the fact that by the very nature of EDC exposure, development is delayed and therefore fish which should be adult by age appear juvenile by morphology. A number of methods have been proposed to deal with this. One uses size classes of male fish and analyses each separately (Doyle et al. 2003). Another includes all fish larger than the smallest fish which are morphologically mature (have a terminal complex of hooks) (Game et al. 2006). In this study we have attempted to define an 'adult male' in a more probabilistic fashion by assigning any fish within the 99% confidence intervals for mean gonopodial length and standard length as a fish which should be an adult. We acknowledge that this method may exclude some EDC-affected fish but we consider this to be the most transparent and rigorous method available.

3.3.5 Vitellogenin

In female mosquitofish, vitellogenin (Vtg) levels were higher in summer (March 2009) than in winter (July 2009) at all WWTP sites, with Vtg only detected in one fish

downstream of Waroona WWTP. While there was little difference in the Vtg measured in females upstream of Harvey WWTP compared with those downstream, higher levels were measured in fish downstream of Waroona WWTP compared with those from the upstream site. Only very low levels of Vtg were measured in female fish from the Serpentine River in summer and no Vtg was measured in fish in winter.

At both the upstream and downstream sites of Waroona WWTP and at the reference site there was negligible Vtg measured in male mosquitofish in both summer and winter. At Harvey, male mosquitofish captured upstream of the WWTP in summer had measureable levels of Vtg but these were lower than that of females from the same population. In winter, male mosquitofish from both upstream and downstream sites of the WWTP had measureable Vtg levels. In winter, at both these sites female mosquitofish had no measurable Vtg levels, so these male Vtg levels represent an induction above female Vtg levels.

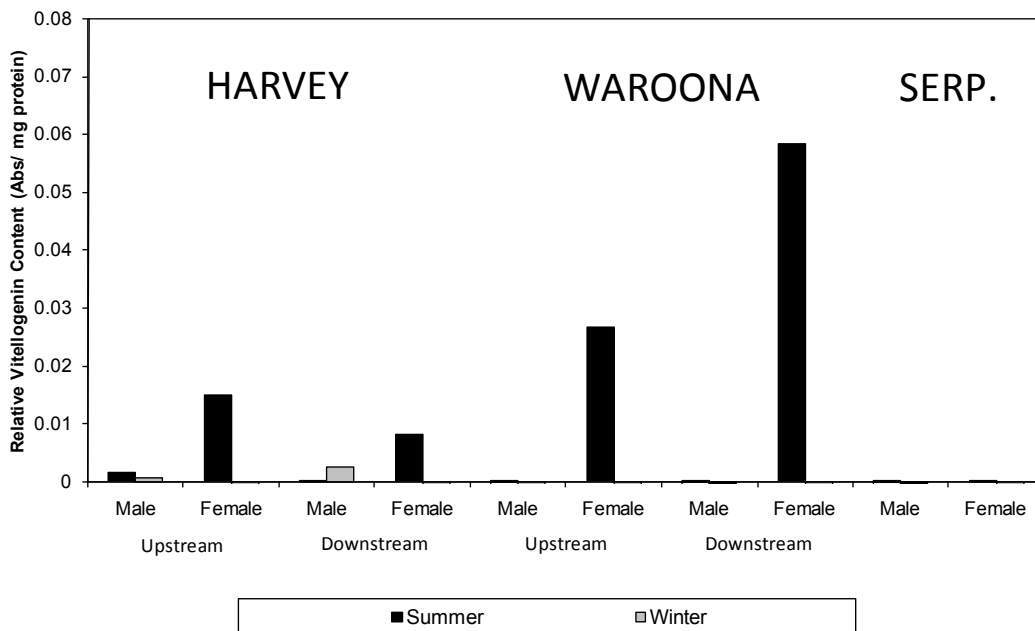


Figure 45: Relative vitellogenin (Vtg) (mean \pm std. err.) content of male and female mosquitofish from the southern WWTP sampling sites during summer (March 2009) and winter (July 2009)

Notes: Vitellogenin content was standardised against the amount of protein in the supernatant sample. Vitellogenin content is expressed as the absorbance at 490 nm standardised against the total protein in the processed sample (Abs/mg protein).

As an egg pre-cursor protein, vitellogenin synthesis is under estrogenic control. Under normal conditions, it is only produced in female fish and only when they are reproductively active. Koya and Kamiya (2000) demonstrated that in *Gambusia* spp. female serum estrogen levels are low outside of the breeding season, from which it

follows that vitellogenin synthesis will also be low. In this study we found that in female mosquitofish vitellogenin was measurable in summer (March 2009) but not in winter (July 2009), suggesting that the fish were not reproductively active during the winter sampling period. This result reflects the fact that mosquitofish use water temperature in combination with day-length as breeding cues, generally breeding from mid-spring to mid-autumn (see Pyke 2006 for a review of reproduction in *Gambusia* spp.). Reproductive threshold temperatures vary geographically though. With winter temperatures at Harvey, Waroona and Serpentine National Park of 16°C, 14°C and 12°C respectively, it would be unusual to find reproductively active female fish. The lack of vitellogenic females at Serpentine during summer (March 2009) is an interesting result and highlights the importance of selecting appropriate reference sites. The temperature at this site would generally be considered sufficient to allow breeding, but the results suggest otherwise. There are two possible reasons for this: either the geographic distance between the WWTP sites and the reference site has imposed differences in threshold temperatures, or the day-length breeding cue is influenced by the high degree of shading and more rugged landscape to cease the breeding period earlier than the more open WWTP sites. In any case, for the purposes of this discussion, breeding had effectively stopped at Serpentine National Park by March 2009.

Male fish carry the gene for the synthesis of the vitellogenin protein but it is not normally expressed except on exposure to estrogenic compounds (Sumpter & Jobling 1995). While female fish have a natural vitellogenin cycle associated with breeding, there is no such cycle in male fish. Therefore levels are solely associated with exposure to estrogenic compounds regardless of reproductive state and should be measurable in summer or winter. The equivalent estrogen concentration range over which male vitellogenin synthesis in a related species (*Gambusia affinis*) occurs has been reported as between 20 and 250 ng/L (Leusch et al. 2005c). However, the LOEC above which vitellogenesis occurs in *Gambusia holbrooki* requires narrowing and confirmation – as do the effects of environmental conditions (e.g. water temperature and day length) on this useful biomarker. At both Waroona WWTP sites and Serpentine National Park (in both summer and winter) there was no measurable vitellogenin content, indicating that at the time of sampling no exposure to estrogenic compounds occurred. This suggests that the equivalent estrogen concentration in water was <20 ng/L. However, at both the upstream and downstream sites of Harvey WWTP, male fish had measureable vitellogenin levels – strongly indicating exposure to estrogenic compounds – and this was the case in both summer and winter (estrogen equivalent concentration in water >20 ng/L). Given that fish from both upstream and downstream of the WWTP effluent discharge point showed this response, it is not possible to attribute this result to the presence of the WWTP. The response is more likely to be due to more diffuse sources of EDCs along the drainage system. To identify these sources, a targeted study of the drainage system covering multiple sampling points across a few kilometres of stream and over different land-use types is required.

3.4 Comparison between the different methods

3.4.1 Comparison of chemical and *in vitro* bioassay analysis

One of this study's objectives was to compare the results of chemical analysis with those of the 'ecotoxicity toolbox' bioassays. Using the concentrations of chemicals and their known potencies in the bioassays, it is possible to calculate a predicted biological activity for each sample. In this study, this could be done for estrogenicity (based on estrogen hormones), androgenicity (based on androgen hormones) and photosynthesis inhibition (based on herbicides, particularly diuron). As previously mentioned, cytotoxicity and genotoxicity can be caused by a wide array of chemical compounds, and it was not possible to relate those bioanalytical measures to individual chemicals.

Predicted estrogenicity was calculated based on the concentration and potency of the natural estrogens 17 β -estradiol, 17 α -estradiol, estrone and estriol and the synthetic estrogen 17 α -ethynylestradiol. When comparing predicted estrogenicity with measured estrogenicity, 48 out of 72 data points (67%) were in good agreement. For six data points the measured activity was higher than predicted from chemical analysis, while for 18 data points the predicted activity was much higher than that measured. It should be noted that 16 of those 18 data points are from Waroona and Harvey WWTPs, where no estrogenic activity could be detected (Figure 38B) even though estrogen hormones (estrone and estriol) were found (Figure 20). The reason behind this discrepancy is unclear. If the Waroona and Harvey datasets are excluded, 30 out of 36 data points (83%) are in good agreement.

The natural hormones 17 β -estradiol and estrone (a metabolite of estradiol) accounted for most ($67 \pm 5\%$) of the predicted estrogenicity.

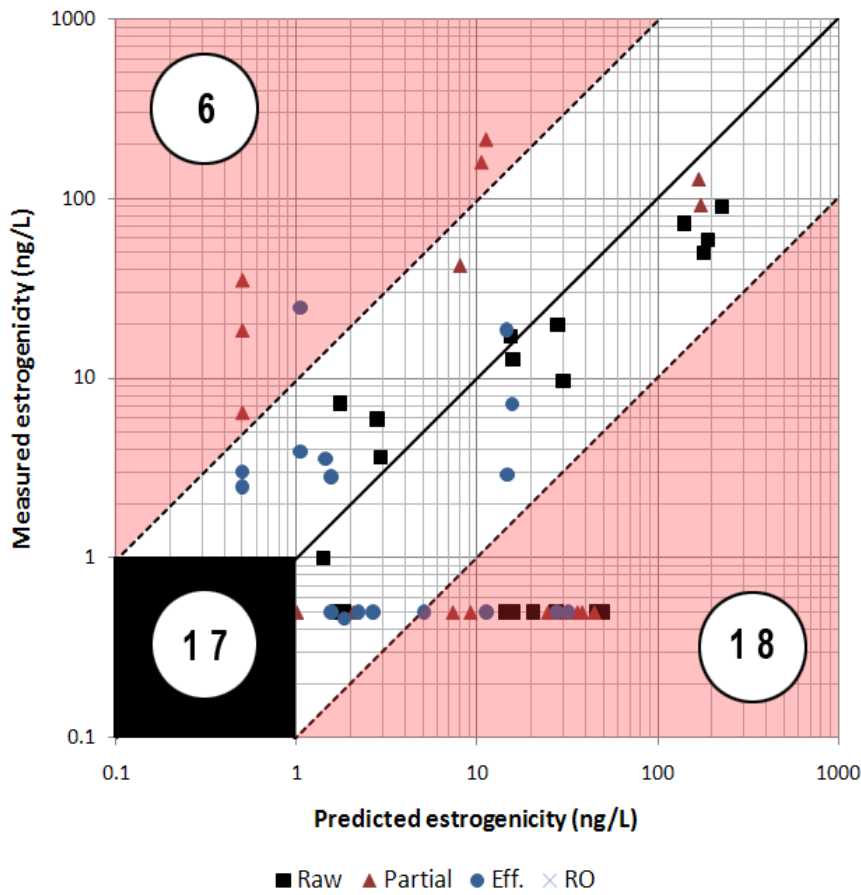


Figure 46: Comparison of measured versus predicted estrogenicity

Notes: The full line represents the isometric line. The areas highlighted in red represent poor agreement between chemical and bioassay analysis (with the number of samples indicated in the white circle). The black area at the bottom left (with 17 samples) indicates good agreement, with both methods quantifying the sample as below detection limit (<1 ng/L EEq).

Predicted androgenicity was based on the concentration and potency of the natural androgens androstenedione, androsterone, etiocholanolone, testosterone, dihydrotestosterone and testosterone propionate. Most samples (47 out of 68, i.e. 69%) were below the quantification limit of the AR-CALUX bioassay (<2.5 ng/L DHT Eq). All of the remaining 21 data points (most of which were raw wastewater) were in good agreement between measured and predicted androgenicity (Figure 47). This suggests that most androgenic chemicals were monitored by chemical analysis. Androstenedione, testosterone and to a lesser extent androsterone were responsible for most of the predicted androgenic activity ($95 \pm 2\%$).

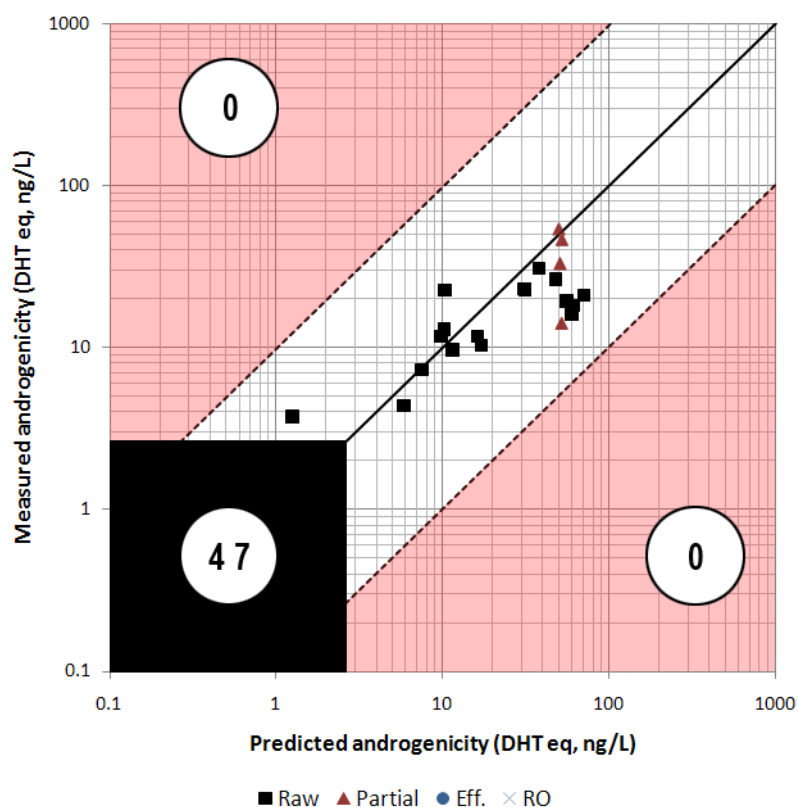


Figure 47: Comparison of measured versus predicted androgenicity

Notes: The full line represents the isometric line. The areas highlighted in red represent poor agreement between chemical and bioassay analysis (with the number of samples indicated in the white circle). The black area at the bottom left (with 47 samples) indicates good agreement, with both methods quantifying the sample as below detection limit (<2.5 ng/L DHT equivalent).

Finally, for photosynthesis inhibition, only diuron concentration and potency were used to calculate a predicted diuron equivalent (DEq). When comparing predicted and measured photosynthetic inhibition, there was generally good agreement (80.7%) between the predicted and measured DEq, with 14 out of 83 data points below the quantification limit of both methods (16.8%) and 53 out of 83 data points in good agreement (63.9%) (Figure 48). There were no samples where the activity was lower than predicted (bottom right quadrant), however measured activity was much higher than predicted for a few samples (19.3%, top left quadrant). This is particularly true for raw wastewater samples, and suggests the presence of other chemicals not accounted for by chemical analysis. As the predicted DEq was based on diuron alone, this is not surprising. It shows that for treated effluent, the remaining diuron is the most significant contributor to photosynthesis inhibition, while other herbicides are likely present in raw wastewater.

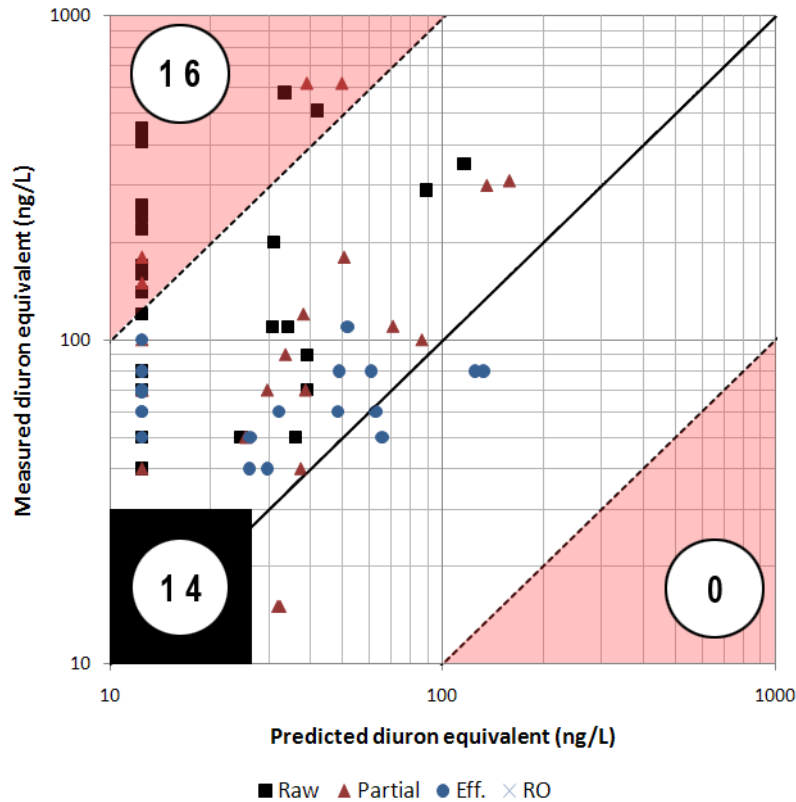


Figure 48: Comparison of measured versus predicted diuron activity (in ng/L)

Notes: The full line represents the isometric line. The areas highlighted in red represent poor agreement between chemical and bioassay analysis (with the number of samples indicated in the white circle). The black area at the bottom left (with 14 samples) indicates good agreement, with both methods quantifying the sample as below detection limit (<25 ng/L diuron for the chemical analysis, <30 ng/L DEq for the bioassay).

3.4.2 Comparison of *in situ* monitoring and WWTP effluent analysis

There is a good correlation between the findings of the *in situ* monitoring and both chemical and *in vitro* bioassay analysis of the WWTP effluents and rivers. Overall, the chemical and *in vitro* bioassay analysis showed good removal of endocrine-active compounds (both estrogenic and androgenic) by the WWTPs to produce effluent with low to undetectable estrogenic and androgenic EDCs. Based on this finding, no significant effect on exposed organisms would be expected, and indeed no significant endocrine-related effects were found in mosquitofish from the receiving environment.

All three lines of evidence (chemical analysis, *in vitro* bioassay analysis and *in situ* whole fish monitoring) suggest that treatment at the WWTPs monitored in this study is adequate to remove the EDCs present in raw wastewater to concentrations where they are no longer of concern.

4 Conclusions and recommendations

4.1 Conclusions

The 'ecotoxicity toolbox' developed in this project has been demonstrated as a useful suite of tools to provide a better understanding of the water quality issues associated with water recycling. The toolbox shows promise for application to water recycling initiatives for a range of end-uses. The following conclusions can be drawn from each line of investigation used in this study:

4.1.1 Bioanalytical methods

- Five bioassay techniques were successfully used to assess cytotoxicity (MicroLumo bioassay), genotoxicity (*umuC* bioassay), photosynthesis inhibition (Max-I-PAM assay) and estrogenic and androgenic endocrine disruption (E-SCREEN and AR-CALUX respectively).
- As expected, there was significant biological activity in raw wastewater, usually higher in the raw wastewater influent to Beenyup AWTP and Gordon Road WWTP compared with Waroona and Harvey WWTPs. Further:
 - there was significant cytotoxicity and phytotoxicity in raw wastewater at all plants
 - there was detectable (albeit low) genotoxicity in raw wastewater at all plants
 - there was significant estrogenicity and androgenicity in raw wastewater at Beenyup AWTP and Gordon Road WWTP, but much less in the raw wastewater of Waroona and Harvey WWTPs (where androgenicity and estrogenicity were often below the detection limit).
- There were differences in removal efficacy between the different plants, with Beenyup AWTP and Gordon Road WWTP generally very effective at reducing the biological activity, while the pond systems at Waroona and Harvey WWTPs were less so. In particular:
 - Cytotoxicity and photosynthesis inhibition were greatly reduced by treatment at Beenyup AWTP and Gordon Road WWTP, but the simpler pond-based systems at Waroona and Harvey WWTPs were less effective. The effluent from all plants was below available guidelines.
 - The oxidation ditch at Gordon Road WWTP produced a marked increase in genotoxicity, which was removed by subsequent treatment steps. Due to the very sensitive nature of the bioassays used, low-level genotoxicity was detected in effluent at Waroona, Harvey and Gordon Road WWTPs, but this activity was not biologically-relevant (below the available guideline).
 - Primary treatment at Beenyup AWTP caused an increase in both estrogenic and androgenic activities, most likely due to reactivation or transformation into more biologically-active compounds.

- Secondary treatment at Beenyup AWTP and the oxidation ditch at Gordon Road WWTP were very effective at reducing estrogenic and androgenic activities.
- Androgenic activity was not detected in any of the effluent samples. Low estrogenic activity was detected in Beenyup AWTP and Gordon Road WWTP effluents, but not in the Harvey or Waroona WWTP effluents.
- RO as part of the Beenyup AWTP was an effective barrier to biologically-active compounds:
 - Genotoxic, phytotoxic, estrogenic and androgenic activity was not detected in RO permeate samples.
 - Low-level cytotoxicity was detected in two out of four RO permeate samples. That activity was almost 20 times less than the available guideline and only 2.7 times higher than the blank. No other specific toxicity was detected in the samples, suggesting that this residual toxicity was not biologically-relevant.

4.1.2 Chemical analysis

- As expected, there were high concentrations of contaminants in raw wastewater. In particular:
 - High concentrations of natural estrogen and androgen hormones were detected in all raw wastewater samples. Wastewater influent at Beenyup AWTP and Gordon Road WWTP usually contained higher concentrations than that of the Waroona and Harvey WWTPs.
 - Diuron was detected in all raw wastewater samples tested.
 - Metolachlor was detected above the ANZECC/ARMCANZ (2000) guideline in Beenyup AWTP raw wastewater.
- Conventional wastewater treatment was generally effective at reducing those concentrations, with a few exceptions. In summary:
 - Conventional wastewater treatment reduced the concentrations of the natural androgens by one to two orders of magnitude, often to below analytical detection limits.
 - Conventional wastewater treatment also reduced the concentrations of the natural estrogens, however simple pond-based treatment plants (Waroona and Harvey WWTPs) appeared less effective than the other two (Beenyup AWTP and Gordon Road WWTP).
 - Diuron was poorly removed by treatment, and was detected in secondary effluent from Beenyup AWTP and effluent from Gordon Road WWTP; albeit below the ANZECC/ARMCANZ (2000) guideline of 0.2 µg/L.
 - Metolachlor was removed by the conventional treatment at Beenyup AWTP, although it was still present at 0.025 µg/L in one instance in secondary treated water (slightly above the ANZECC guideline of 0.02 µg/L).

- RO at Beenyup AWTP was very effective at further removing the contaminants from the water. None of the chemicals monitored in this study were detected in RO permeate.

4.1.3 *In situ* fish monitoring

Upstream and downstream monitoring of mosquitofish (*Gambusia holbrooki*) was conducted at the Harvey and Waroona WWTP discharges.

- The WWTP effluent discharge had no identifiable impact on mosquitofish reproductive morphology at either the Harvey or Waroona WWTPs.
 - Such a lack of impact on an integrative endpoint indicates that any release of EDCs from the WWTP has not detrimentally affected the fish population (as measured by morphological endpoints and demographics).
- There was no observed impact of the WWTP discharge in terms of the shorter-term biomarker of exposure (vitellogenin content in male fish).
- There was evidence of more diffuse sources of EDCs entering the stream to which the Harvey WWTP discharges, with male mosquitofish showing low levels of vitellogenin induction both upstream and downstream of the discharge outlet.

4.1.4 Combining all lines of evidence: the 'toolbox' approach

Chemical analysis and *in situ* monitoring were in agreement with the *in vitro* results: chemical analysis substantiates the removal trends observed by the bioanalytical tools, and *in situ* sampling did not reveal any consistent evidence of endocrine disruption due to discharge of treated wastewater.

This study has demonstrated the utility of combining multiple lines of evidence – chemical analysis, *in vitro* assays and *in situ* monitoring – into a toolbox approach for the assessment of water quality. Biomarkers of exposure and/or effect are complementary and together provide information with a high level of ecological relevance. Nevertheless it is important that the assays are used with careful consideration of confounding factors, and with a high degree of knowledge of study species and environmental conditions.

4.2 Recommendations

This study has demonstrated the usefulness of the 'ecotoxicity toolbox' approach for characterising wastewater quality through the treatment process. Yet several areas need to be further investigated, including:

1. *The role of bioanalytical tools in risk assessment and regulation*

Bioanalytical tools offer clear advantages for monitoring programs because they provide a measure of the biological activity of mixtures, and are a cost- and labour-effective screening strategy. However, there are currently very few bioassay-based guidelines and there is no clear strategy for how to deploy a bioassay toolbox for

monitoring. Further work is needed so that this guidance can be provided to regulators and water resource managers.

2. Further validation of the toolbox and application to other water quality issues

The toolbox approach worked well in the scenario investigated in this study. Further validation of the toolbox against environmental benchmarks and application to other water quality issues would enable testing of how broadly this approach could be applied.

3. Correlation of bioanalytical and chemical tools

Further work is needed to more accurately correlate measured biological activity *in vitro* and predicted activity based on chemical analysis. Discrepancies between the two are particularly interesting and worthy of further investigation, because they may be caused by mixture interaction or the presence of unknown biologically-active compounds.

4. Correlation between in vitro assays and in vivo outcomes

If it can be demonstrated that we can predict *in vivo* impacts using *in vitro* methods, then monitoring programs would be significantly enhanced with respect to high-throughput screening. This has not yet been well demonstrated.

5. Further validation of in vivo and in situ biomarkers and bioindicators

Validation of *in vivo* studies is vital to ensure their utility in accurately describing environmental exposure to EDCs and their effects in wild animals. The first steps toward this validation are laboratory-based investigations linking observed effects with measured EDC concentrations. This should also incorporate development of *in vivo* laboratory screening tests. In addition, attempts should be made to relate these effects in an exotic species (mosquitofish) to native species – which may be more sensitive to EDC exposure given their reproductive physiologies are often different. In a country as large and ecologically diverse as Australia, it is unlikely that a universal model organism will be available, so a number of species that cover a broad geographic range should be included for examination.

6. Investigation of diffuse sources of EDCs

Some male fish in this study exhibited signs of exposure to estrogenic EDCs that were not likely due to WWTP effluent discharges. This suggests that fish may be exposed to other unknown diffuse sources of EDCs. Further work is needed to try to identify the causative chemical(s) and possible land uses and activities behind their release into the environment.

Appendices

Appendix A – Analyte-specific HPLC-MS/MS parameters for estrogens and androgens

Analytes	Retention time (min)	Q1 mass (Da)	Q3 mass (Da)
Oestriol,1	5.3	271.1	253.1
Oestriol,2	5.3	271.1	133
D2-Oestriol,1	5.3	273.216	255.2
D2-Oestriol,2	5.3	273.216	135.2
Trenbolone,1	11.5	271.1	199.2
Trenbolone,2	11.5	271.1	115
Androstendione,1	12.5	287.2	97.1
Androstendione,2	12.5	287.2	109.2
D3-Androstendione,1	12.5	290.197	100.1
D3-Androstendione,2	12.5	290.197	109.1
D417 α -ethynylloestradiol,1	13.1	283.1	135.1
D417 α -ethynylloestradiol,2	13.1	283.1	161.1
D4-17 β -oestradiol,1	13.1	259.1	161.1
D4-17 β -oestradiol,2	13.1	259.1	135.1
D4-oestrone,1	13.1	275.1	161
D4-oestrone,2	13.1	275.1	134.9
Oestrone,1	13.2	271.2	159.2
Oestrone,2	13.2	271.2	133
17 β -oestradiol,1	13.2	255.2	159.3
17 β -oestradiol,2	13.2	255.2	133.2
17 α -ethynylloestradiol,1	13.2	279.2	133.1
17 α -ethynylloestradiol,2	13.2	279.2	159.2
17 α -oestradiol,1	13.8	255.205	159.3
17 α -oestradiol,2	13.8	255.205	133.2
Testosterone,1	14	289.2	97.2
Testosterone,2	14	289.2	109.1
D2-Testosterone,1	14	291.183	99.1
D2-Testosterone,2	14	291.183	111.1
Dihydrotestosterone,1	16.1	291.2	255.2
Dihydrotestosterone,2	16.1	291.2	81.1
D3-Dihydrotestosterone,1	16.1	294.282	258.1
D3-Dihydrotestosterone,2	16.1	294.282	81.1
Etiocholanolone,1	17.3	273.2	255.3
Etiocholanolone,2	17.3	273.2	91.1
D2-Etiocholanolone,1	17.3	275.2	257.1
D2-Etiocholanolone,2	17.3	275.2	215.1
Androsterone,1	17.5	273.2	255.2
Androsterone,2	17.5	273.2	91
Mestradiol,1	19.4	311.058	121.1
Mestradiol,2	19.4	311.058	91.1
Testosterone propionate,1	20	345.1	271.1
Testosterone propionate,2	20	345.1	109.2

Appendix B – Analyte-specific HPLC-MS/MS parameters for herbicides

Name	Q1	Q3	dwel time	DP	FP	EP	CE	CXP	rt
Desisopropyl Atrazine	173.9	104	100	20	95	10	32	16	5.06
Desisopropyl Atrazine conf.	173.9	132	50	20	95	10	25	16	5.06
Desethyl Atrazine	187.9	104	50	20	95	10	35	16	5.86
Desethyl Atrazine conf.	187.9	146	50	20	95	10	25	16	5.86
Simazine	201.9	124	50	20	95	10	23	16	6.81
Simazine conf.	201.9	132	50	20	95	10	28	16	6.81
Atrazine	215.9	96	50	26	90	10	35	16	7.33
Atrazine conf.	215.9	174.1	50	26	90	10	24	16	7.33
Ametryn	227.9	116	50	20	95	10	38	16	7.86
Ametryn conf.	227.9	186.1	50	20	95	10	27	16	7.86
Tebuthiuron	228.7	116	100	12	75	10	36	16	6.97
Tebuthiuron conf.	228.7	172	200	12	75	10	25	16	6.97
Flumeturon	232.7	46	50	12	85	10	38	16	7.18
Flumeturon conf.	232.7	72	50	12	85	10	35	16	7.18
Diuron	234.8	46	200	15	95	10	36	16	7.7
Diuron conf.	234.8	72	200	15	95	10	36	16	7.7
Prometryn	241.7	158.1	50	18	95	10	32	16	8.19
Prometryn conf.	241.7	200.2	50	18	95	10	25	16	8.19
Terbutryn	242.1	91.2	50	20	95	10	39	16	8.31
Terbutryn conf.	242.1	71.1	50	20	95	10	45	14	8.31
Hexazinone	252.8	71	50	8	75	10	45	16	7.24
Hexazinone conf.	252.8	171	50	8	75	10	22	16	7.24
Imidacloprid	256.1	175	50	20	95	10	25	16	5.43
Imidacloprid conf.	256.1	209.1	50	20	95	10	21	16	5.43
Bromacil	261.1	205	100	2	80	4	20	16	6.57
Bromacil conf.	263.1	207	100	2	80	4	20	16	6.57
Metolachlor	252.2	176.2	50	20	105	10	28	16	8.21
Metolachlor conf.	284.2	252.2	50	10	85	10	19	16	8.21
D5 Atrazine	220.9	179.1	50	26	90	10	25	16	7.32

Notes: Q1: Precursor Ion, Q3: Product Ion, dwel time in milliseconds, DP: Declustering Potential, FP: Focusing Potential, EP: Entrance Potential, CE: Collision Energy, CXP: Collision Cell Exit Potential, rt: retention time in minutes.

Glossary

Bioassay	An experimental technique to measure the strength of a biologically-active chemical by its effects on living organisms or their parts (e.g. enzymes, cells).
Bioanalytical tool	A bioassay used to provide a quantitative measure of biologically-active compounds present in a sample, usually performed <i>in vitro</i> in high-throughput format. It is the 'bioassay equivalent' of chemical analysis. In this report, the MicroLumo, Max-I-PAM, <i>umuC</i> , E-SCREEN and AR-CALUX bioassays are proposed as bioanalytical tools.
Bioindicator	A species used to monitor the health of an environment or ecosystem. In this report, the mosquitofish is used as a bioindicator.
Biomarker	A measurable chemical, biochemical, physiological, behavioural or other alteration within an organism. These can be separated into biomarker of exposure (a measure of the internal dose, which may in itself not be particularly health-relevant) or biomarker of effect (a measure of the health effect). In this report, plasma vitellogenin is a biomarker of exposure and population sex ratio is a biomarker of effect.
<i>In situ</i>	Literally 'in the place', describes processes that occur in living organisms in their environment/normal habitat.
<i>In vitro</i>	Literally 'in glass', describes techniques performed in laboratory apparatus rather than in living organisms.
<i>In vivo</i>	Literally 'in living', describes processes that occur in living organisms, or studies examining whole animals as opposed to tissues, organs, cell lines, single-celled organisms, etc.

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