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Endocrine Disrupting Chemicals in the Australian Riverine Environment

A PILOT STUDY ON ESTROGENIC COMPOUNDS

Endocrine Disrupting Chemicals in the Australian Riverine Environment: A pilot study on estrogenic compounds

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A joint project between
CSIRO and Land & Water Australia

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Executive summary

The Endocrine Disrupting Chemicals issue

Certain chemicals in the environment have been shown to interact with the endocrine system of organisms. These compounds are generally referred to as endocrine disrupting chemicals or EDCs. An EDC has been defined as *"an exogenous substance or mixture that alters the function of the endocrine system and can subsequently cause adverse effects in an organism, its progeny or within its (sub)population"* (Damstra *et al*. 2002).

The endocrine system is a complex system which relies on the interplay between a number of chemical messengers, or hormones, to control a number of important bodily functions. EDCs have recently emerged as environmental contaminants of concern due to their ubiquitous nature and their ability to cause hormone-like effects and interfere with normal functioning of the endocrine system in an exposed organism. A number of environmental studies have found evidence of endocrine disruption in wildlife populations, including skewed sex ratios, abnormal development of gonads and reproductive failure in exposed populations of aquatic organisms. Of particular interest are compounds including steroidal hormones, alkylphenols (degradates of non-ionic surfactants), phthalates ("plasticisers"), pesticides and organometals that have endocrine disrupting potency.

Entry of EDCs into the aquatic environment can occur via a number of pathways, such as direct discharge from wastewater treatment plants (WWTPs), paper and pulp mills and intensive livestock operations. Diffuse sources of EDCs can include run-off from agricultural lands treated with pesticides, wastewaters and animal manures. Numerous studies, including some recent work in Australia, have determined a number of these compounds are present in the aquatic environment at concentrations that could potentially be of concern. However, the overwhelming majority of research into the issue of EDCs is being undertaken overseas, particularly in Europe, North America and Japan. Considering the increasing pressure on Australian water resources and the unique fauna that lives within these aquatic systems, it is especially prudent to conduct research that can define the issue of EDCs within Australia.

Therefore, CSIRO and Land & Water Australia (LWA) carried out a joint three-year pilot project to gain a better understanding of occurrence and risks of EDCs in the Australian riverine environment. The report presents part of this work including some significant findings from the project and discusses the implications for future research and management of the EDC issue in Australia.

Project objectives and scope

The principal objectives of this project were:

(a) To provide a review of the international and Australian research on EDCs, the mechanisms involved and the detection methods

(b) To determine factors that can influence the environmental fate of a select group of estrogenic compounds

(c) To survey a number of Australian riverine systems impacted from a number of different activities to assess the levels of a selected group of estrogenic EDCs

(d) To assess the potential for endocrine disruption within the Australian riverine environment using available bioassays, and

(e) To gain a better understanding of the risk EDCs present in the Australian environment and to provide the basis from which future assessments of endocrine disruption in Australian riverine systems could be made.

A number of estrogenic EDCs were selected for analysis based on their estrogenic potency and/or their likely prevalence in effluents. Selected EDCs included the natural and synthetic steroidal hormones estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2) and a number of commonly occurring phenolic xenoestrogens, including nonylphenol mono- and diethoxylates (NP1EO and NP2EO), 4-nonylphenol (NP), 4-*t*-octylphenol (OP) and bisphenol A (BPA). These chemicals have also been the focus of most overseas studies.

Fate of Endocrine Disrupting Chemicals

A number of laboratory experiments assessing the potential fate of these EDCs, in terms of their sorption and degradation (in soils and sediment), indicated that the selected EDCs have a moderate to high affinity for the solid phase especially when high levels of organic carbon were present. In general, the degradation of these compounds was found to be rapid under aerobic conditions, while little degradation occurred under anaerobic conditions. Also, degradation rates of the selected EDCs within sediments with low levels of organic carbon were relatively slower, even under aerobic conditions.

Endocrine Disrupting Chemicals in WWTPs

The scope of this work was reasonably limited, due to the broad nature of the issue and the lack of knowledge relating to it, especially in Australia. Since the endocrine system is complex and EDCs can exert effects on the endocrine system in a number of ways, for this study only estrogenic compounds that exhibit an estrogenic response were selected since this class of EDCs has attracted the majority of research interest. In particular, there has been a lot of interest in steroidal estrogens and a number of xenoestrogens, such as the alkylphenol ethoxylates and BPA. By focussing in this project on these particular estrogenic EDCs in the Australian environment would enable our results to be calibrated against previous international environmental surveys of EDCs.

Surveys of effluents from WWTPs in Queensland, Australian Capital Territory (ACT) and South Australia (SA) were undertaken at two different periods of the year, representing wet and dry seasons for respective areas. The sampling locations also represented sub-tropical (Queensland) and temperate zones (ACT and SA), to assess whether seasonal variations could have an influence on concentrations of target compounds. Along with selected analytical techniques, estrogenic activity in the effluents was measured using a yeast estrogen screen (YES) bioassay.

Concentrations of the selected EDCs were found to be reasonably constant over the two sampling periods both within and between WWTPs. The alkylphenolic xenoestrogens were typically measured at high part per trillion to low part per billion (high ngL^{-1} to low μgL^{-1}) concentrations in the effluent samples. Among the xenoestrogens, BPA and OP had the

lowest concentrations with median concentrations of 21.5 and 39.5 ngL^{-1}, respectively. On the other hand, NP, NP1EO and NP2EO showed concentrations up to two orders of magnitude higher. For example, the concentration of NP ranged from 514 to 2991 ngL^{-1} with a median value of 1113 ngL^{-1}. Similarly, NP1EO and NP2EO were within the same order of magnitude with median concentrations of 1484 and 782 ngL^{-1} for NP1EO and NP2EO, respectively. The steroidal estrogens were found at low ngL^{-1} concentrations, with E1 consistently found at higher concentrations (ranging from 3.1-39.3 ngL^{-1} with a median concentration of 23.9 ngL^{-1}) than E2 (ranging from 0.05-6.3 ngL^{-1} with a median concentration of 3.8 ngL^{-1}), and EE2 (ranging from 0.01-1.30 ngL^{-1} with a median concentration of 0.45 ngL^{-1}).

Based on these concentrations, the steroidal estrogens constituted more than 99 % of the predicted estrogenic activity of the samples. However, the measured estrogenic activity was considerably lower than the predicted estrogenic activity in all samples, indicating mitigation of the estrogenic response ("anti-estrogenicity") within the samples. There are a number of possible reasons for this observed anti-estrogenicity, including variable sampling handling between chemical and biological analytical techniques and the presence of weaker estrogens interfering in the bioassays. Despite these potential limitations, this finding of anti-estrogenicity was further indicated using a series of dilutions of the samples, in the presence of spiked E2, which suggested the need for further investigation to elucidate the source of the anti-estrogenic effect.

The WWTPs in SA represented a range of different treatment technologies, including activated sludge, oxidation ditches and a series of lagoons. Assessment of concentrations of the selected EDCs and associated estrogenic activity was undertaken at a number of different stages of the treatment process to determine the treatment efficiency of each WWTP to remove EDCs. Removal of the phenolic xenoestrogens was highly efficient, except where a series of parallel anaerobic and aerobic lagoons was used. However, removal of the steroidal estrogens was much more variable and none of the treatment technologies used had a clear advantage over another for all steroidal hormones.

For all WWTPs the removal efficiencies of estrogenic activity was more than 92 %, while the measured estrogenicity was again lower than the predicted estrogenic activity. With only the steroidal estrogens contributing to the predicted activity, these findings once more suggested the presence of anti-estrogenic activity in the WWTPs.

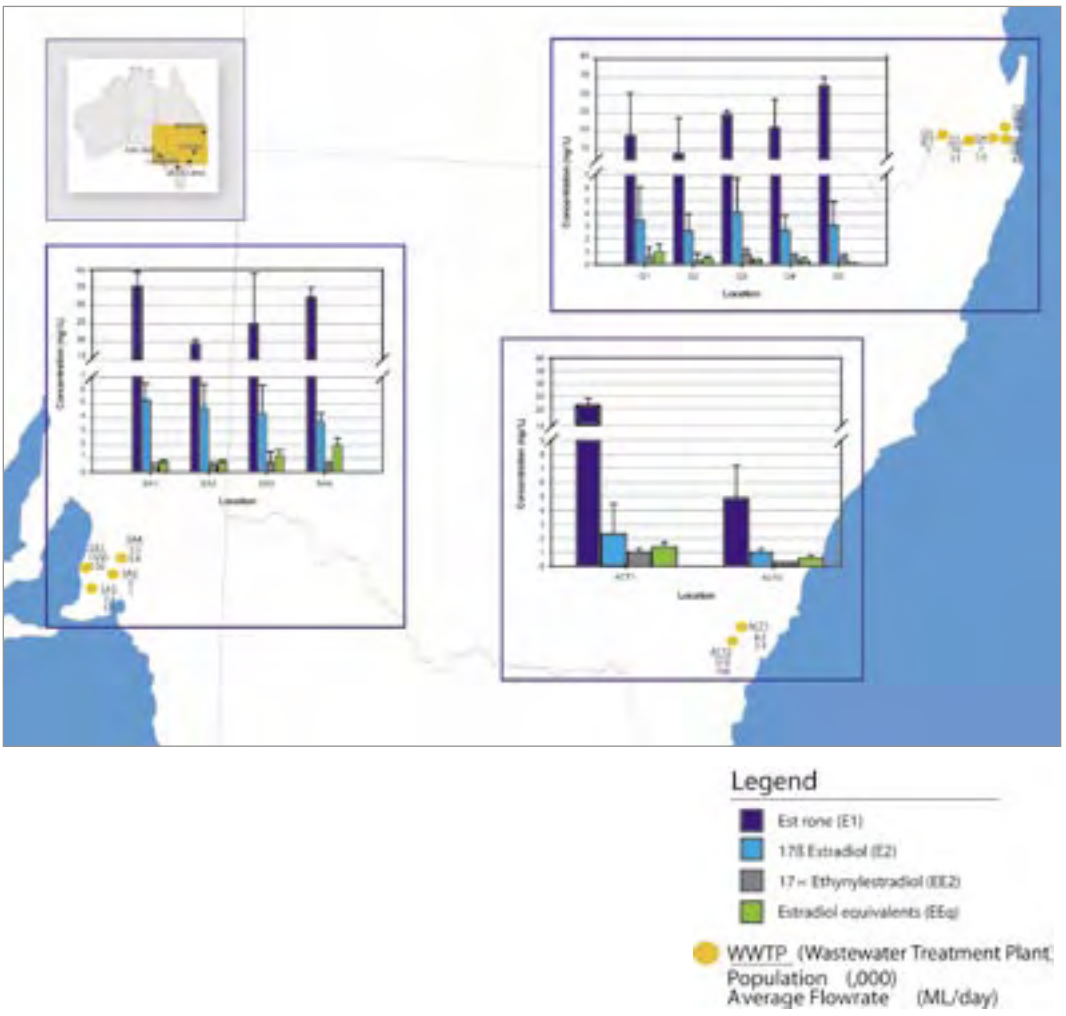
Endocrine Disrupting Chemicals in rural streams

A number of streams in rural regions within SA were surveyed, where land use such as dairy farming, stock grazing and horticulture are practised. For this survey, the naturally occurring E1 and E2 were assessed in water samples using instrumental analytical techniques along with biologically-based assays. The estrogenic activity, was measured by both the YES and estrogen responsive chemically activated luciferase reporter gene expression (ER-CALUX) bioassays.

Measured concentrations of E1 and E2 were similar, in contrast with the surveys of WWTP effluents, and were usually at low ngL^{-1} concentrations. E1 concentrations ranged from 0.17 ngL^{-1} at National Park FC to 38.5 ngL^{-1} in dairy cattle effluent coming from a milking shed (Dairy WF). Concentrations of E1 were usually found to be higher than E2 at each

Figure 1

Mean concentrations of steroidal estrogens and measured estrogenicity in wastewater treatment plant (WWTP) effluent samples from both sampling periods, where estrogenicity is based on E2 equivalents (EEq). Error bars are based on standard deviations of the mean.



sampling site, where E2 concentrations ranged from 0.52 ngL^{-1} at National Park FC to 8.6 ngL^{-1} also from Dairy WF. As with the WWTP survey, the calculated estrogenic activities of the samples were higher than those indicated by both the YES and ER-CALUX bioassays, possibly due to the presence of anti-estrogenic activity within the collected samples.

The results from the survey of rural areas suggest that livestock sources can contribute to the estrogenic load in streams. However, the concentrations of the estrogens, along with potential anti-estrogenic compounds, need to be further considered in terms of the relevance of these values to exposed organisms living within the receiving systems. Also, the relatively high levels noted in selected reference sites warrant further investigation in terms of expected background levels of estrogens and estrogenic activity in Australian riverine systems.

Conclusions and future research needs

The research has identified the specific needs for further research:

- Sorption and degradation are likely to play an important role in the environmental fate of the selected EDCs. This has implications for not only the fate of these compounds following their release into riverine systems but also for water and biosolid re-use and disposal strategies. Furthermore, the study shows that these compounds can be expected at higher concentrations in sediments with potential accumulation under anaerobic environments. The study indicates that application of wastewater on aerobic and biologically-active soils is likely to result in the rapid degradation of the selected EDCs.
- WWTP treatment technology showed variable levels of efficiency in the removal of the selected EDCs. In general, the alkylphenolic xenoestrogens were efficiently removed in all WWTPs, while the removal of the steroidal estrogens was less efficient and varied from WWTP to WWTP. Therefore, removal of the steroidal estrogens was not well predicted by removal efficiencies based on a number of biological parameters that are traditionally used to assess the performance of WWTPs. In terms of removal of the potent steroidal estrogens, research is currently being undertaken into advanced treatment technologies, although low cost treatment options for rural regions also need further consideration.
- Concentrations of selected EDCs were reasonably similar both between sampling locations and sampling periods. These concentrations are likely to be further reduced once dilution occurs in the receiving system and other degradation processes occur within the environment. However, since sorption of these EDCs are likely following their release into a receiving system, any future environmental surveys should measure the concentrations of EDCs in both the water column and sediments. A longitudinal survey of receiving systems should be undertaken to gain a better understanding of the fate and distribution of EDCs within Australian riverine systems. This would be especially critical in aquatic systems where effluent contributes or expected to contribute a large volume to the overall system, such as during dry / drought conditions or with increasing impact of climate change.

- The presence of intensive livestock operations and other animal husbandry operations (e.g. cattle feedlots, dairies and piggeries) in rural areas is likely to contribute to the presence of steroidal estrogens within streams in a particular catchment area. Concentrations of E2 were similar to those found in WWTP effluents, while concentrations of E1 were substantially lower. The presence of these steroidal estrogens in selected reference sites, where no human activity was expected, indicates that more work needs to be undertaken to define background levels of estrogens in Australian riverine systems.
- This study demonstrates the need for combining *in vitro* assays and analytical chemistry for the assessment of EDCs in WWTPs. *In vitro* assays provide an integrative approach for the assessment of estrogenic loads, including metabolites and antagonistic compounds, while chemical analyses enable precise detection and quantification of specific chemicals of concern. Only by combining these two general approaches is it possible to assess both the estrogenic activity in a sample and to (partly) identify and quantify the compounds responsible for the estrogenic activity.
- Any findings from environmental surveys need to be considered in the context of the potential risk of measured concentrations of EDCs to aquatic organisms already living within stressed riverine systems. The YES assays, along with other integrative bioassays, play an important role in defining the potential estrogenicity of waterways. However, the differences in the values predicted by the YES and ER-CALUX bioassays do vary and the final choice of which reporter gene assay to employ (mammalian-based or yeast-based) depends on the importance of lower detection limit versus the importance of ease of use and lower costs. Based on the limited data available, there is no single in vitro reporter gene assay that can be concluded to perform better or to be more reliable than any other assay for screening individual chemicals. While bioassays represent rapid, cheap and convenient screening tools, the development and use of *in vivo* molecular biomarkers, such as vitellogenin induction and gonadal effects in native fish, is critical for future monitoring programmes.
- The integrative nature of the bioassays indicated that there was some extent of anti-estrogenic activity in all of the water samples taken, from both WWTPs and rural streams. There are several possible mechanisms that may contribute to this activity, such as the bioavailability of the compounds to participate in ligand binding in the YES assay, interference from the sample matrix and presence of unknown weak estrogenic or anti-estrogenic compounds. However, without specifically selecting for anti-estrogenic compounds with chemical analyses, it is difficult to determine why this effect is occurring. Because of the potential mitigating influence of anti-estrogenic compounds in waterways, it is important that these compounds be identified and this effect more thoroughly investigated. Future work will also focus on measuring both predicted and measured EEq in the same sample at the sample dilution factor to reduce variability in the data set.
- The presence of the selected EDCs within WWTP effluents are indicative of a wide range of contaminants being released into riverine systems. The presence of these mixtures of compounds needs to be taken into account when further assessment of the fate and effects of EDCs is undertaken. For example, the presence of anti-microbial agents may decrease the effectiveness of the degradation of EDCs both prior to and following release from WWTPs. Also, the effect of EDCs in aquatic organism is usually assumed to be due to additivity of mixture components, compared with synergism or antagonism. These questions relating to mixtures of EDCs needs to be addressed.

This review and research provides new information for those involved in policy, regulation and research investment. The report includes:

- Updates of new developments in scientific research on Endocrine Disrupting Chemicals and their impacts on ecosystems
- Preliminary benchmarks on EDC quantification in Australian effluent and rivers
- Quantification of existing water quality interventions impacts on EDC degradation rates
- Acknowledgement of the role of research investment in the innovations which have occurred in Australia on this issue
- Gains drawn from international EDC research that may be matched in future by sharing policy and regulatory responses, and
- Current gaps in awareness and information provision which could assist with the way civil society manage EDCs.

Contents

Abbreviations

ACT	Australian Capital Territory
APE	Alkylphenol ethoxylate
AR	Androgen receptor
BOD_5	Biological oxygen demand (5-day)
BPA	Bisphenol A
CNS	Central nervous system
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CYP1A	Cytochrome P450 1A enzyme
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DOC	Dissolved organic carbon
DPIW	Department of Primary Industries and Water, Tasmania
E1	Estrone
E2	17b-estradiol
EC50	Chemical concentration at which 50% of test organisms show mortality
EDC	Endocrine disrupting chemical
EE2	17a-ethynylestradiol
EEF	Estrogenic equivalency factor
EEq	17b-estradiol equivalents
ELISA	Enzyme linked immuno sorbent assay
EPA	Environmental Protection Agency / Authority
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERBA	Estrogen receptor binding assay
ER-CALUX	Estrogen responsive chemically activated luciferase reporter gene expression
EROD	7-ethoxyresorufin-O-deethylase
GBRMPA	Great Barrier Reef Marine Park Authority
GC-MS	Gas chromatography-mass spectrometry
hER	Human estrogen receptor
HGELN	HeLa cells-human cervical cancer cells
HPLC	High performance liquid chromatography
HPLC-MS/MS	High performance liquid chromatography triple quadrupole mass spectrometry
K_{oc}	Organic carbon normalised partition coefficient
K_{ow}	Octanol-water partition coefficient
LOEC	Lowest observable effect concentration
LOQ	Limit of quantification
LWA	Land & Water Australia

μgL^{-1}	Micrograms per litre
mgL^{-1}	Milligrams per litre
mRNA	Messenger ribonucleic acid
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
MVLN	MCF-7 breast cancer cell line
ngL^{-1}	Nanograms per litre
NICNAS	National Industrial Chemicals Notification and Assessment Scheme
NP	4-nonylphenol
4-*n*-NP	4-*n*-nonylphenol
NP1EO	Nonylphenol monoethoxylate
NP2EO	Nonylphenol diethoxylate
NSW	New South Wales
NSW DPI	New South Wales Department of Primary Industries
OECD	Organization for Economic Cooperation and Development
OP	4-*t*-octylphenol
PBDE	Polybrominated diphenyl ether
PCNB	Pentachloronitrobenzene
PPCP	Pharmaceutical and personal care product
PPM	Pulp and paper mill
PTFE	Polytetrafluoroethylene
rtER	Rainbow trout estrogen receptor
S	Water solubility
SS	Suspended solid
SA	South Australia
SA EPA	South Australian Environmental Protection Authority
SHBG	Sex hormone binding globulin
SPE	Solid phase extraction
TBT	Tributyltin
TMS	Trimethylsilyl
TOC	Total organic carbon
UPLC-TOF-MS	Ultra-high pressure liquid chromatography time-of-flight mass spectometry
US EPA	United States Environmental Protection Agency
USGS	United States Geological Survey
UV	Ultraviolet
VTG	Vitellogenin
WWTP	Wastewater treatment plant
YES	Yeast estrogen screen
YAS	Yeast androgen screen
ZR	Zona Radiata

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1 The Endocrine Disrupting Chemicals issue

1.1 Endocrine disruption: causes for concern

During the last century, a number of synthetic chemicals were developed for a variety of medical, scientific, agricultural and industrial purposes. Although significant economic and social benefits can be attributed to the use of these chemicals, including an increase in agricultural crop yields and various medical breakthroughs, their widespread use has resulted in the release of many of these synthetic chemicals into the environment (Danzo 1998).

The freshwater environment, rivers and estuaries, are common repositories for large amounts of domestic and industrial waste, principally through wastewater effluents. While the reuse of wastewater is increasing with time in Australia, a large proportion of treated effluent is currently discharged into freshwater or marine ecosystems. In certain circumstances, such as ephemeral streams, wastewater discharged into the receiving environment makes up the majority of the flow, especially in the drier months.

Certain chemicals present in waste water treatment plant (WWTP) effluents have been shown to interact with the endocrine system of wildlife inhabiting the receiving environments. Other contributing sources of chemical contaminants can include agricultural runoff within watersheds from fields fertilised with animal or human manures and natural and synthetic compounds from intensive livestock raising or horticultural practices (Kolodziej *et al*. 2004). These compounds are generally referred to as endocrine disrupting chemicals or EDCs. An EDC has been defined as *"an exogenous substance or mixture that alters the function of the endocrine system and can subsequently cause adverse effects in an organism, its progeny or within its (sub)population"* (Damstra *et al*. 2002). These substances may alter the function of hormonal systems and cause effects by mimicking the effects of natural hormones, blocking their normal action, or by interfering with the synthesis and/or excretion of the hormones (reviewed by Damstra *et al*. 2002).

In response to field observations in the United Kingdom (UK) of hermaphrodite fish in lagoons of a WWTP, a field survey by Purdom and co-workers (1994) was one of the first studies to demonstrate the widespread occurrence of estrogenic compounds in the effluents from WWTPs. This study demonstrated that effluents from WWTPs could contain compounds capable of inducing vitellogenin (VTG) levels in male rainbow trout, *Oncorhynchus mykiss*. VTG is the egg yolk precursor protein which is synthesised in the liver of female oviparous fish under multi-hormonal control, with estrogens playing a dominant role in the regulation of VTG synthesis. VTG production can be triggered in male fish following exposure to estrogens (Sumpter and Jobling 1995). Endocrine disrupting

effects in several wild fish populations living in the receiving waters of WWTP effluents have been reported worldwide, including the United States, Europe, South Africa and Japan (Jobling *et al*. 1998; Allen *et al*. 1999; Sole *et al*. 2000; Folmar *et al*. 2001; Korner *et al*. 2001; Vigano et al. 2001; Gercken and Sorydl 2002; Barnhoorn et al. 2004; Kavanagh *et al*. 2004; Komori *et al*. 2004; Bjerregaard *et al*. 2006). Relatively few studies have been undertaken in Australia, although these also found evidence of endocrine disruption in wild fish populations as well (Batty and Lim 1999; Leusch *et al*. 2006). However, these studies did not assess effects in native species or take into account the diverse nature of the Australian environment.

Alkylphenol ethoxylates, a class of non-ionic surfactants, were initially thought to be responsible for the sexual and reproductive disturbances observed in fish exposed to WWTP effluent due to their relatively high levels detected in the environment and the replication of effects in laboratory exposures at these concentrations. However, recent improvements in analytical selectivity and sensitivity for detecting both natural and synthetic steroidal hormones, such as 17β-estradiol (E2) and 17α-ethynylestradiol (EE2), have enabled their detection at low ngL^{-1} levels in environmental samples (Desbrow *et al*. 1998; Ternes *et al*. 1999a; Snyder et al. 1999; Kolpin *et al*. 2002). Even at these relatively low levels, the steroidal hormones have been implicated in contributing to a significant proportion of the effects observed in the field due to their significantly higher potency (Routledge *et al*. 1998; Snyder *et al*. 2001).

These observations in field studies have led to a significant amount of public interest in the implications of the presence of EDCs in the aquatic environment with respect to their effects on aquatic organisms. This concern has been accompanied by a paradigm shift in the discipline of aquatic toxicology, where chronic exposure to low levels of contaminants has received more attention compared with short-term exposure and effects. For example, low level exposure over a long term, or at least during sensitive periods of an organism's life stage, can have more important implications for the health of a population than acute exposure to higher concentrations of a particular contaminant. The effects of EDCs embody this paradigm shift, in that exposure to previously undetectable concentrations of contaminants in an aquatic ecosystem have the potential to exert profound effects on the normal functioning of an organism's regulatory system.

1.2 Mechanisms of action of EDCs

The endocrine system is a complex system involving several central nervous system (CNS)-pituitary-target organ feedback pathways which are involved in regulating a multitude of functions and maintaining homeostasis (i.e. self-regulated feedback mechanisms within the body). Given this, there are several target organ sites within the endocrine system whereby environmental chemicals could potentially interact and disrupt normal function. Due to the complexity of the cellular processes involved in hormone function, any of these systems may be involved in a chemical's influence on the endocrine system (Henley and Korach 2006). Identity of the specific mode of action of EDCs is also made difficult due to factors including duration, level, type and timing of exposure, cell/tissue type in which the EDC acts and the nutritional status, age and gender of the individual. Consequently, the mechanisms of action of EDCs can be divided into two main categories of receptor mediated and other mechanisms.

- Receptor mediated mechanisms are based on hormones eliciting a response from their target tissue through direct and specific binding to its intracellular or membrane-bound receptors (Tremblay et al. 1998; Soverchia et al. 2005).
- Other mechanisms include those which interfere with the synthesis (Almstrup et al. 2002), release (Ando et al. 2004), distribution and metabolism of hormones, secondary messenger systems (Gillesby and Zacharewski 1998) translational and post-translational mechanisms (LaChapelle *et al*. 2007) and other systems under hormonal control including cytochrome P450 expression (Buhler *et al*. 2000; Montserrat *et al*. 2004).

Interaction with hormone receptors can result in either activation or inhibition of gene transcription by environmental hormones in the following ways:

1. Binding of a compound to a steroidal receptor converts the receptor to an active state, which promotes gene expression (receptor agonist) (Brzozowski *et al*. 1997),
2. Binding of a compound to a steroidal receptor causes the receptor to be unavailable to participate in gene expression (receptor antagonist) (Brzozowski et al. 1997; Tremblay *et al*. 1998),
3. Receptor phosphorylation, where activation of receptors occurs through processes not involving receptor binding (Daniel *et al*. 2007).

Changes in gene expression represent a critical step in the regulation of normal biological function, including cell proliferation and differentiation responses essential for the normal development and function of organ systems (Crisp *et al*. 1998; Damstra *et al*. 2002). For example, steroidal sex hormones, such as E2, elicit their action through regulating gene transcription by binding to receptors that interact with specific DNA sequences (White *et al*. 1994). Those EDCs that are either estrogen or androgen receptor agonists or antagonists have received the greatest attention, partly due to their importance during embryonic development. However, endocrine disruption through pathways other than the estrogen and androgen receptors has been less studied. Other mechanisms include disruption of thyroid hormone dependent metamorphosis in the African clawed frog, *Xenopus laevis*, exposed to Clophen A50 (technical PCB mixture) (Gutleb *et al*. 2007), alteration in tissue distribution and thyroid levels in rats exposed to polybrominated diphenyl ether (PBDE) (Kuriyama *et al*. 2007) and imposex in the neogastropd *Nucella lapillus* exposed to tributyltin (TBT) mediated through the Retinoid X receptor signal pathway (Castro *et al*. 2007).

1.3 Classes and sources of EDCs

An extensive and diverse number of compounds have been identified as EDCs which have been shown to exhibit endocrine disrupting effects both *in vitro* and, to a lesser extent, *in vivo*. Generally, these compounds can be divided into three broad categories:

1. Natural compounds, which are required within a normally functioning endocrine system. Endogenous steroidal hormones include the estrogens, such as E2 and estrone (E1), and androgens, such as testosterone; plant-derived phytoestrogens include genistein, β-sitosterol and coumestrol.
2. Synthetic steroidal hormones are compounds intentionally designed to target the endocrine system including estrogens, 17α-ethynylestradiol and diethylstilbestrol, and antiestrogens, such as tamoxifen.

3. Synthetic chemicals, which are designed for a variety of uses, have been found in some cases to have disrupted the endocrine systems of wildlife and humans. This group represents the most diverse range of compounds and includes pesticides, organohalogens, organotins, alkylphenol ethoxylates (as well as degradation products), BPA, heavy metals and phthalate esters (Safe and Gaido 1998; Baker 2001).

EDCs are released from a wide variety of both point sources including discharges from industry, WWTPs, pulp and paper mills (PPM), intensive animal husbandry (e.g. cattle feedlots, dairies and piggeries) as well as diffuse sources including runoff from agriculture and leaching from landfills (Ying and Kookana 2002; Kolodziej *et al*. 2004; Hutchins *et al*. 2007). Endogenous steroidal hormones and synthetic chemicals in aquatic systems are principally derived from point sources, such as WWTPs, PPM and animal feedlot effluents, although diffuse sources can also contribute to inputs. Table 1.1 contains an overview of key EDCs, both natural and synthetic, including their sources and types of effects they may cause.

1.4 Effects of EDCs: worldwide laboratory and field observations

Of the research undertaken on EDCs worldwide, a considerable amount of effort has gone into assessing the effects that can be elicited by EDCs and at what concentrations these effects occur. In general, field-based work has found the endocrine disruption that can occur when aquatic organisms are exposed to complex mixtures that are likely to contain EDCs, such as WWTP effluents. Conversely, laboratory-based work, while defining effects relating to endocrine disruption, tends to focus on concentrations of EDCs that can elicit an effect and avoiding the complexity of environmental samples.

1.4.1 Laboratory studies

A significant amount of research effort has focussed on the effects of EDCs relating to aquatic organisms including invertebrates such as waterfleas and snails (Baldwin et al. 1995; Oehlmann et al. 2000; Olmstead and LeBlanc 2000) and vertebrates such as frogs and, in particular, fish (Kloas et al. 1999; Lange *et al*. 2001; Metcalfe *et al*. 2001; Qin et al. 2007). Laboratory studies with fish have demonstrated various endocrine disrupting effects ranging from VTG protein induction, altered sex ratios to intersex, which can be induced by estrogenic substances known to be present in effluents at environmentally relevant concentrations (Lange *et al*. 2001; Metcalfe *et al*. 2001; Thorpe *et al*. 2003; Nash *et al*. 2004; Van den Belt *et al*. 2004). Direct comparisons between studies are often difficult due to non-standardised approaches, although natural steroid hormones have been reported to cause effects related to endocrine disruption at ngL^{-1} levels. For example, a study by Tabata *et al*. (2001) demonstrated that long-term exposure (200-230-d until pre-maturity) of Japanese medaka (*Oryzias latipes*) to E2, at concentrations detected in the environment (5 ngL^{-1}), caused individuals to produce ova-testis or abnormal gonads and altered sex ratios. Due to the estrogenic potency of E2, the estrogenic potential of all other estrogenic EDCs are reported relative to E2 (see 3.1 Potency of EDCs).

Table 1.1
Examples of key EDCs, their sources and types of effects observed in laboratory studies

Classification	Compounds	Sources	Examples of biological effects	References
Natural hormones	17β-estradiol Estriol Estrone Testosterone	WWTP [a] effluent	Induce testis-ova in fish, induce ZR [b] and VTG [c], alteration of natural hormone levels, and reproductive failure in fish	Kang *et al*. 2002 Chikae *et al*. 2004 Knoebl *et al*. 2004 Nash *et al*. 2004 Barucca *et al*. 2006
Phytoestrogens	β-sitosterol Zearalenone Coumesterol Lignans Isoflavonoids Genestein	PPM effluent [d], ingestion	Bind to ERs [e] (estrogenic, antiestrogenic), inhibit proliferation mechanisms, alter steroid production	Collins *et al*. 1997 Strauss *et al*. 1998 Almstrup *et al*. 2002
Pharmaceuticals	17α-ethynylestradiol Tamoxifen Mestranol Diethylstilbestrol	WWTP effluent, ingestion	Agonistic and/or antagonistic binding to ERα, ERβ, VTG regulation, reproductive failure in fish	Chikae *et al*. 2004 Nash *et al*. 2004 Van den Belt *et al*. 2004 Ortiz-Zarragoitia & Cajaraville 2005
Alkylphenols	Nonylphenols Pentaphenols Octylphenols Nonylphenol ethoxylates Butylphenols	WWTP effluent, point sources	Reductions in reproductive success, effects on egg production, testis-ova, developmental effects, VTG induction and androgen decreases in fish, affects sexual differentiation in amphipods	Gray & Metcalfe 1997 Brown *et al*. 1999 Gray *et al*. 1999 Toomey *et al*. 1999 Kang *et al*. 2003 Soverchia *et al*. 2005 Balch and Metcalfe 2006
Phenols	Bisphenol A Bisphenol F	WWTP effluent	Agonistic binding to ER, VTG induction in fish, altered sex ratios in frogs	Kloas *et al*. 1999 Kwak *et al*. 2001 Yamaguchi *et al*. 2005
Phthalates	Di-*n*-pentyl phthalate Di-ethylhexyl phthalate Di-hexyl phthalate Butyl benzyl phthalate Di-*n*-butyl phthalate Dicyclohexyl phthalate	Leaching from plastics - ubiquitous	Agonistic binding to ERα, antagonistic binding to ERβ and AR [f]	Zacharewski *et al*. 1998 Takeuchi *et al*. 2005
Organotins	Tributyltin Triphenyltin	Antifouling paint on ship hulls	Imposex, intersex, increased testosterone levels, bivalve larval development, bioaccumulation in gastropods, oysters	Gooding *et al*. 1999 Hwang *et al*. 1999 Alzieu 2000
Organohalogens	Dioxins 2,4-Dichlorophenol Furans Polychlorinated biphenyls	PPM effluent, ubiquitous	Sex reversal in fish and turtles, gonadal abnormalities, CYP1A [g] induction	Crews *et al*. 1995 Matta *et al*. 1998 Smeets *et al*. 1999
Polyaromatic hydrocarbons (PAHs)	Benzo(*a*)pyrene Retene Benz(*a*)anthracene Pyrene 6-hydroxy-chrysene Phenanthrene	PPM effluent, ubiquitous	Antiestrogenic, VTG inhibition, embryo teratogenicity, CYP1A induction	Navas and Segner 2000 Oikari *et al*. 2002 Billiard *et al*. 2004 Hodson *et al*. 2007
Pesticides	Atrazine, Lindane Chlordane, Malathion Dicofol, Permethrin Dieldrin/Aldrin Simazine, Endosulfan Toxaphene, Trifluralin Heptachlor, Kepone Vinclozolin	Agriculture, WWTP effluent, ubiquitous	Intersex in fish and frogs, VTG production, alterations in serum hormone levels in frogs	Palmer and Palmer 1995 Metcalfe *et al*. 2000 Hayes *et al*. 2002
Heavy Metals	Arsenic Lead	Point sources, ubiquitous	Transgenerational effects in fish, reduced fertilisation and altered sex ratios	Matta *et al*. 2001

a wastewater treatment plant; **b** zona radiata **c** vitellogenin; **d** pulp and paper mill effluent; **e** estrogen receptor; **f** androgen receptor; **g** cytochrome P450 1A enzyme

1.4.2 Field studies

In wildlife populations, associations have been reported between reproductive and developmental effects and exposure to EDCs (Baker 2001). One of the most extensively studied and best-documented cases that synthetic chemicals may be capable of impairing the reproductive function in reptiles was in a study with alligators (*Alligator mississippiensis*) in Lake Apopka, Florida. In 1980, following a major spill of an organochlorine pesticide dicofol, there was a significant decline in the number of juvenile alligators in Lake Apopka. Futhermore, female alligators had twice the plasma concentration of estradiol and abnormal ovarian morphology, while male alligators had abnormally small phalli and poorly organised testis, along with a 25% reduction in testosterone levels (Guillette *et al*. 1994; Guillette *et al*. 1996; Harrison *et al*. 1997; Crain *et al*. 1998). Another well documented case of endocrine disruption occurred in marine invertebrates, where masculinisation of female neogastropod snails ("imposex") was reported as early as 1970. Since then available evidence shows that over 100 species of gastropods worldwide have been affected by imposex, rendering them unable to reproduce in extreme cases, due to exposure from tributyltin (TBT), an anti-fouling paint used on ship hulls (Horiguchi *et al*. 1995; Harrison *et al*. 1997; Matthiessen and Gibbs 1998; Gooding *et al*. 1999; Alzieu 2000).

The evidence available for the disruption of reproductive function in wild animals exposed to certain EDCs has been reported in various species including, invertebrates, fish, reptiles, birds and mammals (including humans), with the majority of studies coming from the aquatic environment (Foster 1998; Tyler *et al*. 1998). Endocrine disrupting effects including altered hormone and specific protein levels (Hashimoto et al. 2000; Sepulveda *et al*. 2004), intersex (both male and female reproductive organs) (Jobling *et al*. 2002; Bjerregaard *et al*. 2006), imposex (females with male sex organs) (Reitsema and Spickett 1999; De Wolf *et al*. 2001), reduced reproductive success (Jobling *et al*. 2002) and abnormal growth or reproductive development (Batty and Lim 1999; Gross *et al*. 2001; Kavanagh *et al*. 2004). For most of the reported effects in wildlife, the evidence for a causal link with endocrine disruption is still weak or non-existent, although in some instances a more definitive link has been made (Kavlock *et al*. 1996).

A report published by Canadian scientists is perhaps one of the most significant recent field studies on the low-level chronic exposure of wild fish populations to steroidal hormone. Kidd and co-workers (2007) carried out a seven year, whole lake experiment at the Experimental Lakes Area in north-western Ontario, Canada. In this study, fathead minnows (*Pimephales promelas*) were exposed to low concentrations (5-6 ngL^{-1}) of EE2, added three times weekly during the open water seasons of 2001-2003 for a period of 20-21 weeks. The exposure led to the feminisation of males indicated by the production of VTG mRNA and protein, intersex in males and altered oogenesis in females. In fact, the exposure ultimately caused a near population collapse of this fish from the spiked lake. This study clearly demonstrates that freshwater environmental concentrations of estrogens can seriously impact the sustainability of some wild fish populations under continuous exposure conditions.

1.5 EDC evaluation: common *in vitro* and *in vivo* approaches

Due to the subtle nature of effects that EDCs have been linked to following environmental exposures, there has been a paradigm shift from traditional toxicological assessments which focus on short-term effects, such as cell or whole organism death, to more subtle endpoints, such as protein induction and reproduction. In response, a number of *in vitro* assays and *in vivo* assays have been designed to evaluate the subtle effects of EDCs and, in particular, the estrogen mimicking compounds or xenoestrogens.

1.5.1 *In vitro* testing

A number of *in vitro* assays have been developed to screen for chemicals and waters (including WWTP effluents) for estrogenic / anti-estrogenic and androgenic / anti-androgenic activity. These assays discriminate chemicals that are capable of binding to the steroid receptors and, in some cases, transcriptional activation. The most commonly used *in vitro* assays used for screening estrogenic or androgenic potentials are listed in Table 1.2. These *in vitro* assays differ in their sensitivities, applications and endpoints, with each assay having various advantages and disadvantages in their use and the information which they provide. A group of commonly used *in vitro* assays include the reporter gene assays, which include the yeast estrogen screen (YES) and the estrogen responsive chemically activated luciferase reporter gene expression (ER-CALUX) assays. The advantages of the reporter gene assays include the use of eukaryotic cells, can distinguish between agonist and antagonist (in most cases), their cost-effectiveness, ease of use and, due to their extensive validation, can be standardised relatively easily for inter-laboratory comparisons. Some disadvantages of the reporter gene assays, particularly with the YES assay, include genetic drift over time of test cells, bioavailability of analytes to the cells and do not always distinguish between agonists and antagonists (Zacharewski 1997; Beresford *et al*. 2000).

Table 1.2
Examples of *in vitro* tests for assessing the effects of EDCs

In vitro assay	Test	Distinguishes	Endpoints	Reference
Competitive ligand binding assays	ER[a]α, ERβ, AR [b]	Receptor agonists	Relative binding affinity to ER, AR	Soto *et al.* 1998
Cell proliferation	E-screen	ER receptor agonists and antagonists	Cell proliferation	Soto and Sonnenschein 1995
Recombinant receptor/reporter gene assays	YES [c], ER-CALUX [d], MVLN [e] cells, HGELN [f] cells	hER [g], rtER [h], AR, agonists and antagonists	Transcriptional activation	Routledge and Sumpter 1996 Gutendorf and Westendorf 2001 Van den Belt *et al.* 2004 Legler *et al.* 2002
Protein based assays	Trout hepatocytes	ER, prolactin, SHBG [i], EROD [j], VTG [k]	Up-down regulation	Toomey *et al.* 1999 Laville *et al.* 2004

a estrogen receptor; **b** androgen receptor; **c** yeast estrogen screen; **d** estrogen receptor chemical activated luciferase expression (human T47D breast cancer cells); **e** MCF-7 breast cancer cell line, **f** HeLa cells-human cervical cancer cells, **g** human estrogen receptor, **h** rainbow trout estrogen receptor, **i** sex hormone binding globulin, **j** ethoxyresorufin-O-deethylase, **k** vitellogenin

1.5.2 *In vivo* testing

While *in vitro* assays are useful from a mechanistic standpoint and as screening tools, *in vivo* assays permit the detection of effects that result from multiple mechanisms and also take into account processes such as bioavailability, toxicokinetics, metabolism and "cross-talk" between biological pathways (Zacharewski *et al*. 1998). Measuring the effect of EDCs in whole organisms such as invertebrates, amphibians, fish and birds has advantages over using cellular in vitro assays (Campbell *et al*. 2006). For example, assays at the whole organism level can assess population effects, life-cycle, fecundity, deformities, growth, reproductive deficiencies and offspring development (Brown *et al*.1999; Bowman *et al*. 2000; Ferguson *et al.* 2000; Kelly and Di Giulio 2000; Matta *et al*. 2001; Yokota *et al*. 2001; Segner *et al*. 2003).

In the OECD and US EPA, programmes have been established for screening EDCs. The US EPA is currently developing and validating *in vitro* and *in vivo* assays to determine the potential for chemicals to cause endocrine disruption in humans or wildlife. To achieve this goal the US EPA is using a two-tiered approach. The first tier involves a screening battery and is intended to identify chemicals affecting the estrogen, androgen or thyroid hormone systems through any of the recognised modes of action. Whereas, tier 2 testing is intended to confirm, characterise, and quantify those effects for estrogen, androgen and thyroid active substances in invertebrates, amphibians, fish, birds and mammals (USEPA 2007).

2 EDCs in Australian environments

Identification of the likely sources of EDCs in Australian environments can be informed by surveys of WWTPs which have been conducted in several countries, including the US, UK, Germany, Netherlands, Denmark, Ireland and Japan. These surveys have reported the occurrence of certain EDCs in WWTP effluents and their receiving environments. The general trends that can be found across these studies includes the choice of target compounds (natural and synthetic steroidal estrogens and estrogenic alkylphenol ethoxylates and their alkylphenol metabolites) and the levels at which they have been detected. Generally the steroidal hormones are selected as they pose the greatest risk due to their potency, whereas the alkylphenol ethoxylates and their alkylphenol metabolites, despite their weaker estrogenicity are chosen due to their prevalence and relatively high concentrations. However, it is commonly reported that the steroidal hormones are responsible for the majority of the estrogenicity of the effluents (› 90 %) due to their relatively higher estrogenic potential (Snyder *et al*. 2001; Korner *et al*. 2001; Aerni *et al*. 2004; Rutishauser *et al*. 2004). Although variation does exist between studies, most work has found that the natural and synthetic steroidal hormones are detected in treated effluent in the low ngL^{-1} range, whereas the alkylphenols and alkylphenol ethoxylates are sometimes detected at several orders of magnitude higher than this.

2.1 Potential sources in the Australian riverine environment

Although there is a paucity of information relating to the presence of EDCs in the Australian riverine environment, the pattern of release into these systems is expected to follow that of other countries. Assessment of endocrine disruption in freshwater systems has been undertaken to a limited extent in Australia, although a number of studies have found evidence of endocrine disruption in the marine environment due to human impacts, such as though release of organotin antifouling agents (Nias *et al*. 1993; Reitsema and Spickett 1999). However, the limited work undertaken in Australia would suggest that a number of EDCs are present in freshwater systems in urban, peri-urban and rural areas. In Australia, the effects of EDCs on behaviour of certain fish species in Sydney basin has been demonstrated by Dr Richard Lim, co-workers and others (e.g. Batty and Lim 1999; Doyle and Lim 2005; Leusch *et al*. 2006).The presence of EDCs is both evidenced by analytical techniques targeting specific compounds and biological assays assessing the overall endocrine disrupting potential (usually as estrogenic activity) of water samples.

2.1.1 Urban environments

The most important pathway of EDCs into freshwater systems is from WWTP effluents. WWTPs receive wastewater from a diverse range of sources. Sewage from residential

areas, waste from industries using high quantities of surfactants and wastewater from hospitals, can all contribute to the overall levels of EDCs. More than 5000 ML of wastewater effluent is generated from WWTPs each day in Australia, with the majority being discharged into aquatic receiving systems (Ying and Kookana 2002). One study in a freshwater system in western Sydney receiving WWTP effluents indicated that endocrine disruption was likely to be occurring, based on the observations made on male mosquitofish, *Gambusia affinis holbrooki*, collected in the area (Batty and Lim 1999).

Biosolids, which are separated from wastewater during the treatment process, can contain appreciable quantities of contaminants that are also present in aqueous waste. This could be an issue if biosolids are applied to land that is part of water catchment areas for freshwater systems where leaching plays an important transport role. Surveys of Australian WWTPs effluents have shown mean concentrations of E1 up to 54 ngL^{-1}, E2 up to 19 ngL^{-1} and a number of other xenoestrogens up to 589 ngL^{-1} (Braga et al. 2005; Leusch *et al*. 2005; Tan *et al*. 2007). Furthermore, the assessed wastewater effluents were found to have levels of estrogenicity, measured by the E-screen bioassay, up to 68 ngL^{-1} E2 equivalents (EEq), indicating a relatively high level of estrogenic activity (Leusch *et al*. 2005; Tan *et al*. 2007). Based on the relative potencies of estrogens (see 3.1 Potency of EDCs), the concentrations of the steroidal estrogens indicate they would be of most interest, in terms of their potential to cause endocrine disruption, and probably contributed to the majority of the measured estrogenicity.

2.1.2 Rural environments

Intensive agricultural and animal husbandry operations could potentially be an important source of EDCs in the Australian environment. Intensive agricultural practices in areas can lead to pesticide contamination of freshwater systems through run-off and spray drift. For example, the presence of the organochlorine pesticides DDT and toxaphene in the Ord River in north-west Australia were found to lead to the bioaccumulation of these pesticides within estuarine crocodiles, *Crocodylus porosus* (Yoshikane *et al*. 2006). Incidences of surface water contamination by pesticides in Australia have been previously reviewed by Kookana *et al*. (1998) and AATSE (2002).

Intensive livestock raising, such as dairy and beef cattle, swine and poultry, may also be an important contributor of EDCs through run-off and wastewater (Kjaer *et al*. 2007). In this case, naturally occurring steroidal estrogens, such as E1 and E2, are likely to contribute to the majority of the estrogenicity. Surveys from a number of European countries reported estrogenic activity in watersheds that were previously supposed to be unaffected by anthropogenic inputs. It was concluded that intensive livestock practices in the surveyed areas were likely to be the principal source of the measured estrogenicity (Matthiessen *et al*. 2006).

In Australia, some pulp mills are located in rural areas and discharge effluents into aquatic ecosystems. There have been a number of studies reporting evidence of endocrine disruption, including feminisation and masculinisation, in waters receiving effluent from pulp mills (McMaster *et al*. 1995; Cody and Bartone 1997; Mellanen *et al*. 1999). The mixture of chemicals found within these effluents can be quite diverse, with chlorinated organic compounds (e.g. dioxins), surfactants (e.g. alkylphenyl ethoxylates and their metabolites) and naturally-occurring phytosterols (e.g. β-sitosterol) released from wood during processing (Ying and Kookana 2002; Manning 2005). Therefore, it has been difficult to elucidate which compounds in particular are responsible for, and to what extent agonistic and antagonistic interactions play a role in, the observed endocrine disruption.

3 Ranking of important EDC groups

Due to the large diversity of compounds that are suspected of or have been found to causing endocrine disruption, most research in this area has focussed on EDCs that are likely to present the greatest risk once released into the environment. Selection of EDCs is usually undertaken by ranking EDCs based on a hazard assessment approach. For a hazard assessment the potency of a compound is compared against the likely exposure concentrations of the compound in the environment. Therefore, the perceived hazard of an EDC is based on both environmental concentrations and concentrations of exposure that can lead to endocrine disruption ("effective concentrations").

Most effort regarding endocrine disruption research has focussed on estrogenicity. This has included the relative estrogenic potency and the concentrations of EDCs that elicit an estrogenic response, both *in vivo* and *in vitro*. Also, a number of environmental surveys have been undertaken to selectively measure the concentrations of estrogenic compounds within treatment plants and environments that are likely to receive estrogenic compounds. These surveys have especially focussed on the steroidal estrogens and phenolic xenoestrogens.

3.1 Potency of EDCs

The potency of EDCs, relative to a benchmark compound, is used to assess their potential to bind to biological receptors. In the case of estrogenic compounds, the steroidal hormone E2 is often used as a benchmark for the estrogenic potential of a compound.

The estrogenic potential of EDCs is usually determined from in vitro estrogenicity screening, such as the YES, ER-CALUX and MVLN assays. However, there is often variability between these assays, while *in vivo* assessment can further complicate the prediction of endocrine-disruption potential (Marlatt *et al.* 2006). For example, EE2 was found to be 25 times more potent than E2 *in vivo* compared with in vitro assessments (Van den Belt *et al.* 2004). However, steroidal estrogens are consistently found to be many orders of magnitude more potent than phenolic xenoestrogens, such as akylphenols and BPA (Table 3.1).

Table 3.1
List of EDCs ranked as being most significant due to their relative potency to E2, concentrations detected in WWTP effluent and observed biological effects

Class	Compound	Relative Potency to E2 (*in vitro*)	[WWTP effluent] (ngL^{-1})	Example of reported biological effects (*in vivo*)	Concentration of effects (ngL^{-1})
Natural hormones	17β-estradiol (E2)	1.0 [a]	1-48 [c]	Reduced sexual behaviour and impregnation success in adult male mosquitofish, *Gambusia holbrooki* [g]	20 (84 d)
	Estrone (E1)	0.22 [a]	1-76 [c]	VTG induction in adult zebrafish, *Danio rerio* [h]	204 (21 d, EC50)
Pharmaceuticals	17α-ethynylestradiol (EE2)	1.03 [a]	0.2-7 [c]	Skewed sex ratios and ova-testis in fathead minnow, *Pimephales promelas* [i]	4 (305 d)
Phenols	Bisphenol A (BPA)	0.0001 [a]	< 1000 [e]	Altered sex ratios in African clawed toad, *Xenopus laevis*, larvae exposed from 2 days post-hatch [j]	23 (84 d)
Alkylphenols	4-*tert*-octylphenol (4-*t*-OP)	0.0004 [a]	nd-500 [e]	Reproductive malformations (additional female organs) in adult freshwater snails, *Marisa cornuarietis* [k]	1 000 (140 d)
	4-*n*-nonylphenol (4-*n*-NP)	0.000009 [a]	<170-37000 [f]	Induction of VTG in immature juvenile Atlantic salmon, *Salmo salar* [l]	50 000 (7 d)
	4-nonylphenol (4-NP)	0.00014 [a]			
	Nonylphenol ethoxylates (NP1EO, NP2EO)	0.000006 [b]	nd-332000 [f]	Intersex condition in Japanese medaka, *Oryzias latipes* exposed from 1 day post-hatch [m]	300 000 (100 d)

a based on yeast estrogen screen (Woods 2007), **b** (Jobling and Sumpter 1993), **c** (Desbrow *et al*. 1998), **d** (Rudel *et al*. 1998), **e** (Blackburn and Waldock 1995), **f** (Snyder *et al*. 1999), **g** (Doyle and Lim 2005), **h** (Van den Belt *et al*.2004), **i** (Lange *et al*. 2001), **j** (Kloas *et al*. 1999), **k** (Oehlmann *et al*. 2000), **l** (Meucci and Arukwe 2005), **m** (Balch and Metcalfe 2006), nd-not detected, VTG-vitellogenin.

3.2 Effective concentrations

Assessment of the estrogenicity of EDCs has been more commonly determined, with respect to effective concentrations. Concentrations of less than 100 ngL^{-1} have been found to induce VTG production, alter sex ratios and the abnormal formation of gonads in the Japanese medaka, *Oryzias latipes* (Tabata *et al*. 2001; Nilsen *et al*. 2004). Also, exposure of the male mosquitofish, *Gambusia holbrooki*, to 20 ngL^{-1} E2 for nearly 3 months altered sexual behaviour and reduced success of impregnation (Doyle and Lim 2005).

The synthetic steroidal estrogen EE2 has been found to cause significant effects in a number of fish species at concentrations less than 10 ngL^{-1}. Impacts include VTG induction, abnormal gonadal development and skewing of sex ratios (Lange, *et al*. 2001; Nash *et al*. 2004; Ortiz-Zarragoitia and Cajaraville 2005). Also, exposure to up to 6 ngL^{-1} of EE2 caused the near collapse of a fathead minnow (Pimephales promelas) population in a Canadian lake (Kidd *et al*. 2007). On the other hand, much higher concentrations of alkylphenols (greater than 1 μgL^{-1}) are required to cause similar effects in certain fish species (Gray *et al*. 1999; Hemmer *et al*. 2001; Metcalfe *et al*. 2001) (see Table 3.1).

3.3 Environmental concentrations

The majority of environmental surveys previously undertaken have focussed on concentrations within the water column. Within freshwater systems steroidal estrogens have been found at concentrations less than 100 ngL^{-1} and as low as 0.2 ngL^{-1} in the case of EE2, although typical concentrations of steroidal estrogens are in the low ngL^{-1} range (Table 3.1). Alkylphenols, alkylphenol ethoxylates and bisphenol A (BPA) are usually found at concentrations greater than 1 μgL^{-1} and up to 330 μgL^{-1} in the case of the nonylphenols (Table 3.1).

The presence of steroidal estrogens in aquatic systems has generated the most interest in environmental assessment of EDCs. Although their concentrations are quite low, their relative potencies have demonstrated that these concentrations within aquatic systems is cause for concern. Alkylphenols and BPA, although with considerably lower relative estrogenic potency, have also been detected at substantially higher concentrations in aquatic systems receiving effluents (Table 3.1). The use of the hazard assessment approach indicates that the measured concentrations in the environment are at levels that could potentially lead to endocrine disruption in aquatic organisms. The analytes selected were, therefore, based on this hazard assessment, as well as findings from extensive literature reviews (Ying and Kookana 2002; Ying *et al*. 2002b) and discussions with experts from Australia and New Zealand.

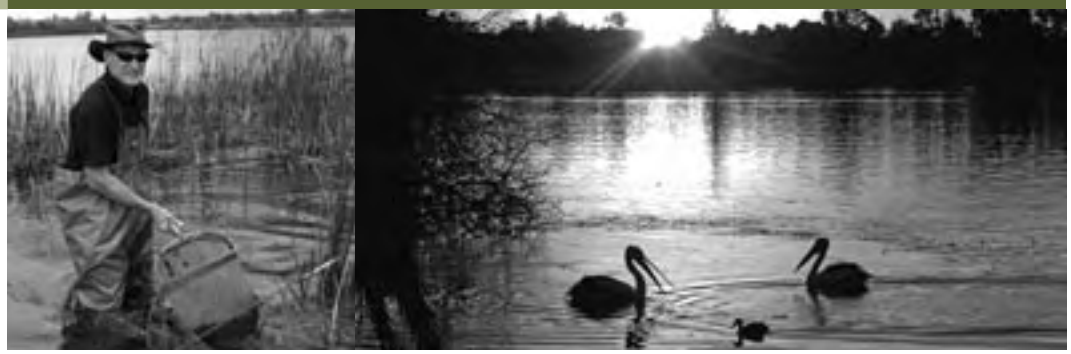
3.4 EDC effects: difficulties in prediction

In the past decade, there has been an extensive amount of research conducted regarding endocrine disrupting effects in the environment (Sumpter and Johnson 2005). However there are still gaps in the research knowledge that make it difficult to predict the effects of EDCs on organisms that still need to be addressed:

- There remains a lack of baseline data. That is, little information exists for what is considered normal in many wild populations and test species. To increase the confidence of concluding whether the observed response is a result of EDC exposure, several considerations need to be made, including the use of large sample sizes, seasonal changes (spawning), life-history, comparison of effects to reference populations of the same species, reproduction of effects under controlled laboratory conditions, consideration of widespread effects by inclusion of multiple species (Hashimoto *et al.* 2000; Jobling *et al.* 2002; Nash *et al.* 2004; Hutchinson *et al.* 2006).
- Continuous exposure to low doses is the most likely scenario in the aquatic environment where contaminants constantly enter receiving waters at low concentrations (such as WWTP effluent). A large body of evidence exists, from both field and laboratory studies, demonstrating that low ngL^{-1} concentrations of EDCs are able to cause effects if the exposure is continuous (Jobling *et al.* 1998; Folmar *et al.* 2001; Lange *et al.* 2001; Barnhoorn *et al.* 2004; Bjerregaard *et al.* 2006). Compounds such as E2 and EE2, while less prevalent in the environment, are potent estrogens and released on a continuous basis (Sumpter and Johnson 2005). Also, many compounds identified as xenoestrogens are lipophilic, which can lead to bioaccumulation in continuously exposed organisms (Soverchia *et al.* 2005). Structurally diverse compounds present in WWTP effluents, such as alkylphenols and BPA, are weakly estrogenic and although they may only play a minor role in the estrogenic load of the effluent, the long term health effects of low concentrations of these chemicals on the reproductive physiology of fish is poorly understood (Jobling *et al.* 1998).

- Metabolites or degradation by-products formed from the transformation of the parent compound can still be potent EDCs. For example, transformation of E2 by oxidation leads to the formation of the relatively less potent E1, although E1 is still considered to be a potent EDC (D'Ascenzo *et al.* 2003). Alternatively, degradation of NPEO in the environment leads to the formation of the more potent EDC NP (Ying *et al.* 2002b; Jobling and Sumpter 2003). Therefore, the loss of EDCs through transformation does not necessarily lead to a loss of endocrine disrupting activity or can potentially even lead to an increase.
- Organism sensitivity can be highly variable, meaning the concentration required to exert a biological response varies depending on certain factors including age, nutritional status, organism health, target tissue type and potency of the EDC that the organism is exposed to (Tyler *et al.* 1998). Although the biochemical pathways involved in hormone action are similar in all vertebrates, the effects at the physiological and cellular response level may differ. For example, in male birds differentiation of the testis is influenced by E2 whereas in mammals it is under control of testosterone (Ankley *et al.* 1998).
- Multiple mechanisms of action infers that EDCs can exhibit multiple modes of action which can either be concentration- or tissue- or ligand-dependent. Predicting the effects of these types of compounds is difficult. For example, differential effects of phthalate esters on transcriptional activities (via human ERs a and b) has been reported, where some phthalates were not only ERa agonists but ERb antagonists (Takeuchi *et al.* 2005).
- Chemical mixtures in wastewater effluents can lead to organisms being simultaneously exposed to a multitude of chemicals capable of disrupting the endocrine system. Laboratory studies have shown that the combined effects of steroidal estrogens can be additive, so even when the concentration of the steroidal estrogens is below the lowest observable effect concentration (LOEC), the combined mixture can still cause an effect (Silva *et al.*. 2002; Thorpe *et al.* 2003; Brian *et al.* 2005). However, predicting the effects of chemical mixtures may be difficult when interactions between chemicals cause combination effects to deviate from what is expected based on individual compounds (Kortenkamp and Altenburger 1998). For example, deviations from additive behaviour (where effects of each individual compound in the mixture add up to the overall effect) are possible due to the effects of xenoestrogens acting through multiple pathways, as well as modifications of chemicals through interactions with other components of the mixture (Gaido *et al.* 2003).

4 Project overview - Assessment of EDCs in the Australian riverine environment



The principal objectives of the CSIRO / LWA EDC research project were:

(a) To determine factors that can influence the environmental fate of a select group of estrogenic compounds;

(b) To survey a number of Australian riverine systems impacted from a number of different activities to assess the levels of a selected group of estrogenic EDCs;

(c) To assess the potential for endocrine disruption within the Australian riverine environment using available bioassays;

(d) To gain a better understanding of the risk EDCs present in the Australian environment and to provide the basis from which future assessments of endocrine disruption in Australian riverine systems could be made.

4.1 Scope of this project

The presence of EDCs in aquatic ecosystems is still an area that is being actively researched internationally and, the acquired knowledge relating to their fate and effects, and how this relates to the risk they present to ecosystem health, is growing rapidly. Since the vast majority of current research has been conducted in Europe, North America and Japan, the paucity of information relating to EDCs in the Australian environment presents a stark contrast. When this work was initiated, little information was available relating to the EDCs in the Australian environment, i.e. at what concentrations the common EDCs are present and how these may affect native fauna under Australian conditions.

The numbers of compounds that have been identified as potential EDCs are likely to be in the hundreds, or even thousands. Therefore, as previously discussed, the compounds were selected based on a suite of commonly occurring compounds that have been identified in literature as potent EDC plus our own assessment from literature review and consultation with stakeholders. The compounds selected for this project included steroidal estrogens (E1, E2 and EE2), alkylphenol ethoxylates (NP1EO and NP2EO), alkylphenols (OP and NP) and BPA. Although EE2 is strictly a xenoestrogen, being a synthetic compound, from here on xenoestrogen will be used to refer to only the alkylphenols, alkylphenol ethoxylates and BPA.

Therefore, this project was undertaken to provide a limited synopsis of the state of EDCs in the Australian environment. Riverine systems were selected to represent a system that is not further confounded by tidal and significant dilution effects (as in estuarine/ marine receiving systems). Surveys of riverine systems were a snapshot of conditions at a specific time and place and the systems selected represented a broad overview of receiving systems.

The project's objectives were addressed through laboratory-based assessments of environmental fate of EDCs in sediments and soils (including both extent of sorption and degradation) and assessment of estrogenic loads in riverine systems, using a number of screening tools. Water samples from both rural and urban riverine systems, with a range of potential sources of EDCs, were analysed for estrogenicity and concentrations of selected EDCs.

Despite the limited resources this project was designed to ensure that the information generated from it is able to provide a basis for future work on EDCs in the Australian environment. The riverine systems selected for the surveys were from both temperate and sub-tropical climates, during periods of the year when the influence of external environmental parameters, such as rainfall and temperature, would be captured. A number of WWTPs were selected to determine the effectiveness of a number of different treatment regimes could be compared. Also, both rural and urban systems were selected to assess whether different sources of EDCs can influence the estrogenic potential of receiving waters and the concentrations of the selected compounds of interest.

4.1.1 Environmental fate

Understanding the fate of EDCs once they are in the aquatic system provides important information relating to the potential distribution and persistence within the environment. This information can be used for planning environmental surveys and assessment of the environmental risk of EDCs. For example, if a compound of interest is found to be below limits of detection in the water column, this may indicate that it is either quickly degraded or being distributed to other phases, such as sediments. Assessment of environmental fate therefore provides additional information that can address such unknowns.

The sorption to soils and sediments of the selected EDCs was undertaken to assess their potential distribution once they are released into the aquatic environment. Also, the stability of the selected EDCs was assessed in soils and sediments, within both aerobic and anaerobic environments. The degradation study was undertaken to address the stability of these compounds in water and to microbial degradation.

4.1.2 Environmental concentrations

A survey of streams receiving inputs from WWTPs, in both rural and urban settings, was undertaken in Queensland, South Australia and the ACT targeting the selected EDCs. The WWTPs included a number of treatment processes, such as activated sludge, biological filters and a series of anaerobic / aerobic lagoons. Also, the number of people that the WWTPs serviced varied considerably, with populations ranging from 3 300 to 1.3 million people, with corresponding average daily flow rates ranging from ‹ 1 to › 100 ML.

Therefore, a range of climate zones, wastewater treatment processes and sources of contaminant input were selected to assess whether these factors could influence the levels of the selected EDCs in a number of Australian riverine systems. This assessment would give important baseline information, which can be compared with international studies and give a further indication of the potential risk that these EDCs may pose to Australian systems.

Rural streams where livestock (including dairy and grazing) and horticultural practices were part of the watershed were compared within a stream with an urban watershed. In this study, the steroidal estrogens E1 and E2 were quantified and converted into an estrogenic equivalent (EEq), based on the relative potencies and concentrations of E1 and E2. At each of these sites, an EEq was also determined using in vitro bioassays, including the ER-CALUX and the YES assays.

Analytical tools, including gas chromatography-mass spectrometry (GC-MS) and enzyme-linked immunosorbent assay (ELISA), were used for these surveys.

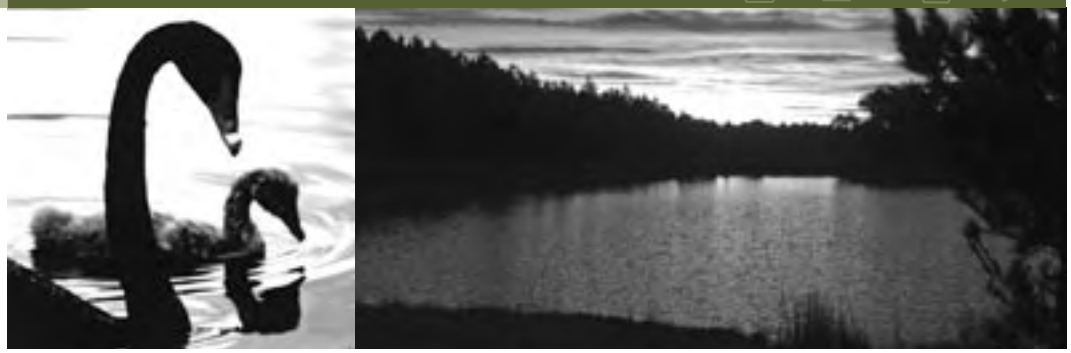
4.1.3 Estrogenicity of effluents

Water samples were assessed for their estrogenic and androgenic potential using the YES and ER-CALUX assays.

The advantages of the YES assay include the lack of endogenous ER in the yeast *Sacchromyces cerevisiae* and the relative sensitivity, ease and cost of the assay. The YES bioassay accounts for the total estrogenicity of the medium, compared to the selective nature of specific analytical methods, such as GC-MS and ELISA. Furthermore, the antagonistic effects of anti-estrogens can be evaluated using the bioassay. However the *in vitro* nature of the assay does not account for bioavailability, metabolism and cell membrane transport mechanisms that add complexity to estrogenic / androgenic responses *in vivo*.

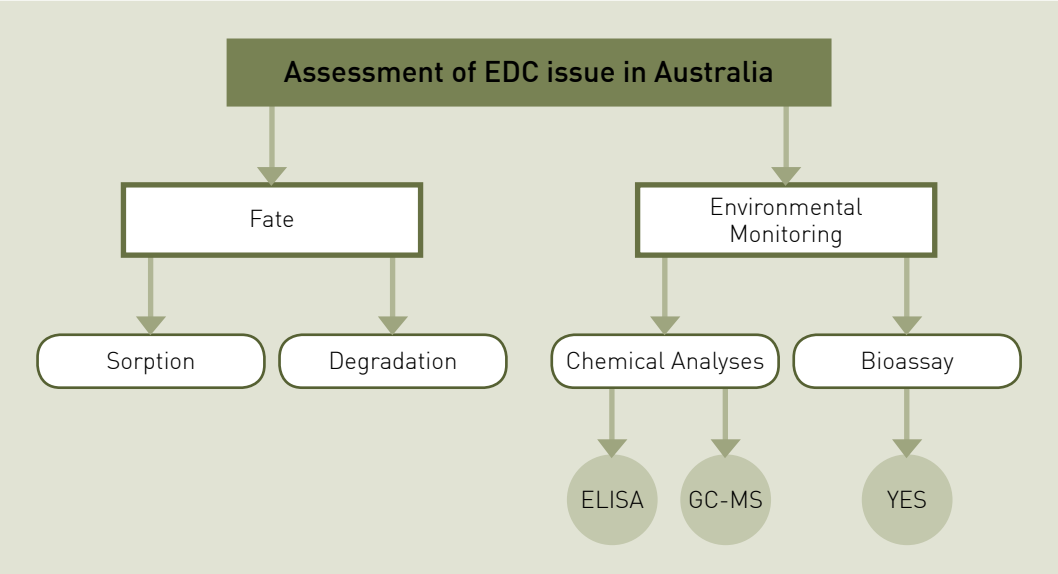
Therefore, as a general screening tool the YES assay is useful in evaluating the potential overall estrogencity of a test medium. Further investigation regarding causality of *in vivo* effects must be undertaken using specific bioassays or analytical techniques.

5 Methodologies



This section gives an overview of the approaches undertaken during laboratory experimentation and environmental surveys. An outline of sampling strategies, experimental and analytical methods and quality assurance / quality control measures are presented in this section (Figure 5.1).

Table 5.1
Schematic diagram showing an overview of the research method



5.1 Environmental fate of EDCs

The transport, distribution and fate of EDCs in the environment may be influenced by many processes taking place in the soil and aquatic environments, including sorption, desorption and degradation. In this section, the sorption and degradation of selected EDCs was assessed within model systems containing both a solid and an aqueous phase.

5.1.1 Sorption

Studies were undertaken to assess sorption of the steroidal estrogens, as well as BPA, OP and NP on four soils (from sandy to clay), and biodegradation of five of the EDCs (BPA, E2, EE2, OP, NP) in a loam soil associated with wastewater reuse. Sorption coefficients of the seven EDCs on the four soils were determined by using a batch equilibrium method (Ying and Kookana 2005).

Four soils (RC, RF, TF and WT) and an aquifer sediment used in this study were collected at a depth of 0 to 15 cm from agricultural land in South Australia. Selected soil and water properties were determined (Table 5.1 and 5.2). Soil pH was measured in a 1:2.5 w/v soil to 0.01M $CaCl_2$ solution using a pH meter, while soil organic carbon was determined by a LECO carbon and nitrogen analyzer. Soil particle size distribution was analysed by using the pipette method (U.S. Department of Agriculture 1996).

Soil suspensions in glass bottles contained initial concentrations of 50 μgL^{-1} for NP and 25 μgL^{-1} for the remaining compounds. Samples were mechanically shaken for a pre-determined equilibration time of 2 h before being centrifuged for 30 min. The collected supernatant solutions were analysed for selected EDCs using high pressure liquid chromatography (HPLC) with online solid phase extraction (SPE) and a fluorescence detector (Ying *et al.* 2002a).

The sorption coefficients for each compound were calculated using the Freundlich isotherm where a range of concentrations were used in the batch sorption experiments. The Freundlich isotherm was determined from the relationship:

$$K_f = \frac{C_s}{C_{aq}^n}$$

where K_f is the Freundlich sorption coefficient, C_s is the concentration in the solid phase, C_{aq} is the concentration in the aqueous phase and n is the linearity of the isotherm. Where $n \approx 1$ the relationship can be given as:

$$K_d = \frac{C_s}{C_{aq}}$$

where K_d is the sorption coefficient.

5.1.2 Degradation

Biodegradation of the E2, EE2, BPA, OP and NP was characterised in the sandy loam, RF, and also aquifer sediment (Table 5.1). The biodegradation experiments were performed under aerobic and anaerobic conditions. The nominal concentration of each EDC applied to the soils was 1 μgg^{-1}. The incubation temperature for all experiments was 20°C. All manipulations for the anaerobic degradation experiments were undertaken inside an anaerobic incubation chamber. The concentrations of all five EDC compounds were monitored on days 0, 1, 3, 7, then weekly until 70 days. All experiments were performed in duplicate with duplicate sterile controls monitored at the same times. The above protocols provided an opportunity to assess both biotic and abiotic pathways of loss under both aerobic and anaerobic conditions.

Samples (and sterile controls) were extracted twice by shaking for 2 h with 20 mL of ethyl acetate. The extracts were evaporated to dryness under a nitrogen stream and redissolved in 20 μL of acetonitrile. The extracts were derivatised, an internal standard added and analysed by GC-MS.

The disappearance of the target analyte was related to the initial amount of the analyte within the soil, which was given the arbitrary value of 1. The degradation half-life ($t_{1/2}$) was reported based on the following first-order relationship:

$$t_{1/2} = \frac{\ln 2}{k}$$

where $\ln 2$ is the natural logarithm of 2 (0.693) and k is the slope of the plot of amount (relative to 1) against time.

Table 5.1
Physicochemical properties of soil and sediment used in fate studies

Soil	pH	TOC [a] (%)	Clay (%)	Silt (%)	Sand (%)
Sediment	8.9	0.5	3	1	83
RC	7.2	1.3	12	2	85
RF	5.4	0.85	10	4	84
TF	7.4	1.8	48	23	27
WT	5.6	2.9	25	20	52

a total organic carbon

Table 5.2
Physicochemical properties of water samples used in fate studies

Water sample	pH	NO_3-N [a] (mgL^{-1})	PO4-P [b] (mgL^{-1})	DOC [c] (mgL^{-1})
Aquifer	7.9	0.03	0.02	1.5
Creek	7.9	0.02	0.02	7

a nitrogen as nitrate; **b** phosphorus as phosphate; **c** dissolved organic carbon

5.2 Environmental levels of EDCs

The environmental surveys utilized a range of analytical tools including bioassays, an immunoassay and chromatographic-mass spectrometric techniques to evaluate the total loading of selected EDCs, as well as chemical identification of selected EDCs in the environment. It should be noted that chemical analysis of the steroidal estrogens was only selected based on the actual compound, rather than attempting to distinguish the conjugated forms. Excretion of steroidal estrogens from the human body is facilitated by conjugation of these compounds with more water soluble groups, such as glucuronide and sulphate (Belfroid *et al*. 1999; Stuer- Lauridsen *et al*. 2005). Entry into a WWTP can lead to deconjugation, where the conjugated group is removed and the parent compound can again be detected using analytical techniques (Panter *et al*. 1999; Ternes et al. 1999; Baronti *et al*. 2000). A study by the Danish EPA suggested the majority of estrogens (usually › 90 %) detected in effluents are in the unconjugated form (Stuer-Lauridsen *et al*. 2005).

5.3 Sampling

The sampling sites for this project included various riverine environments receiving effluent sources that were likely to contain estrogenic EDCs, based on findings from previous international studies. These sites included Waste Water Treatment Plants (WWTPs) and streams receiving effluent from areas of intensive livestock in a number of Australian regions.

5.3.1 Waste Water Treatment Plant locations

WWTPs selected for the survey included those in the Australian Capital Territory (ACT), Queensland (Q) and South Australia (SA). Two ACT sites (namely ACT1 and ACT2), five Queensland sites (namely Q1-Q5) and four SA sites (namely SA1-SA4) were chosen for this survey (Table 5.3). Therefore, these sampling areas included both temperate and sub-tropical regions. The sites were selected in consultation with local authorities and collaborating agencies such as the Queensland EPA, SA EPA and Ecowise Environmental. The access to these sites was generally granted on the condition of anonymity and these sites have been referred to by code. Rural and peri-urban streams receiving discharges from wastewater treatment plants (WWTPs), dairies and stock grazing catchment areas were also included. Assessment of pesticides were not included in this study since, although widely used, several monitoring programmes by state EPAs and other natural resource management agencies (SA EPA; DPIW Tasmania; GBRMPA) are being conducted and considerable data is already available in literature on pesticides (Kookana *et al*. 1998; AATSE 2002).

5.3.2 Effect of treatment technologies

The fate of selected EDCs at different stages in the treatment plants were investigated by analysing concentrations of EDCs before, during and after the treatment. These studies provided an assessment of removal of EDCs during treatment process under a set of varying treatment technologies.

Four WWTPs from South Australia (SA1, SA2, SA3 and SA4) were chosen in this study and sampling was undertaken in July 2005. SA1 is a large municipal activated sludge treatment plants with a tertiary treatment stage of six lagoons. Final effluent from SA1 is discharged into the sea or reused for irrigation. The other three plants are small rural WWTPs. Final effluents from the three rural WWTPs are discharged to small creeks. SA2 uses a biological process with two oxidation ditches followed by chlorination. SA3 uses activated sludge process with three bioreactors followed by UV disinfection and chlorination. SA4 comprises ten lagoons operated in series consisting of two parallel anaerobic lagoons followed by eight aerobic lagoons (Table 5.3).

5.3.3 Temporal and spatial variation of EDCs

The environmental monitoring for EDCs at various sites in the three states was carried out over at least two seasons. This aim was to assess the variations in the concentrations of the selected EDCs in the effluents and receiving environments at different times. The sampling times represented winter and summer in the sub-tropical and temperate sites. The time of sampling was expected to have some bearing on the levels of EDCs in effluent due to (i) the variability in flow volumes, due to rainfall patterns and (ii) the ambient conditions (e.g. temperature) potentially impacting the rate of degradation of compounds during treatment.

In South Australia the samples were collected in July 2005 and December 2005. In ACT the wastewater samples were collected from two effluent schemes in September 2004 and February 2005. In Queensland, the samples were collected in October 2004 and March 2005. In all cases, the former sampling period represented winter sampling, with high rainfall in the temperate zones and low rainfall in the sub-tropical WWTPs. Conversely, the latter sampling periods represented high temperatures and low rainfall in the temperate zones and high rainfall for Queensland. Therefore, in the temperate climates of ACT and SA, the two seasons differed markedly in ambient temperatures and levels of rainfall. In contrast in Queensland, the levels of rainfall vary markedly between seasons, while the variation in mean temperatures was small.

5.3.4 Rural sampling sites

To investigate the animal sources of estrogens in riverine environments, locations were selected from three major dairy regions in South Australia that were expected to represent the worst case concentrations of estrogen that were likely to be present in the environment from dairying activities. The locations of the sampling sites are given in Figure 5.2.

Sites from irrigated dairying on the lower River Murray (RM) floodplain included wash-down water from a milking shed (Dairy WF) and two large drains that collected irrigated runoff from a number of dairy operations in the Wallflat and Jervis region (Drain WF and Drain JD). The samples from the dairy shed were collected directly from an overflow pipe, representing unchlorinated washdown water from a typical milking shed operation in the area. A site upstream of dairying was sampled from the River Murray just upstream of the township of Mannum to act as a control (Upstream RM). Another site was sampled from the River Murray just downstream from all dairying activities at Jervois to show whether the cumulative impact from dairying produced a detectable concentration in the major river in the State (Downstream RM).

The Myponga River on the Fleurieu Peninsula was sampled to provide an indication of background effects from typical dairy cattle grazing in the region. Sites were selected to include a location where stock had direct access to the creek (Blocker's Road Dairy - BD), a site that had been fenced to prevent stock accessing the creek as part of a major riparian protection programme (Rogers Road - RR) and a reference site at Emerald Hill in the upper catchment in an area of native vegetation where no stock were present (EH).

The third dairy region sampled was from the South East. Sites sampled included Mosquito Creek (Dairy MC), where irrigated dairy cattle had access to the stream. Also, two sites on Stony Creek were selected, where one site was just downstream from a major irrigated dairy on the margins of Lake Bonney SE (Downstream SC). The other site at Stony Creek (Upstream SC) was a catchment area that drains agricultural grazing area, mainly related to sheep grazing.

Additional samples were also taken from another seven streams to provide an indication of the presence of estrogens in the wider environment from a selection of stream types found in the southern part of the State. Samples were collected from three streams with stock grazing (sheep and/or beef cattle) as a major land-use in their catchments, including Scott Creek (Creek SC), Bremer River (River BR) and Finniss River (River FN). Also, four streams without stock grazing that included a creek draining an irrigated horticultural catchment at Lenswood (Horticulture LC) and an urban stream at Dry Creek (Urban DC) and streams within two conservation parks at First Creek (National Park FC) and Rocky River (National

Park RR) The sites in the conservation parks were considered to be free from any anthropogenic impacts and were used as reference sites for this study. All selected sites were part of an ambient water quality monitoring programme for the South Australian EPA and knowledge about the general water quality, biological condition and land use of the sites were all factors in their selection.

Table 5.3
Wastewater treatment plant (WWTP) parameters from selected sites, including population serviced, average daily water flows and treatment processes used.

Region	WWTP	Population serviced [a]	Average daily treatment volume (ML d^{-1})	Treatment process	Additional treatment
ACT [b]	ACT1	8 214	2.3	Oxidation ponds	Stabilisation lagoon
	ACT2	310 000	100	Activated sludge	Cl2 [c]
Queensland	Q1	70 000	11	Biological nutrient removal	-
	Q2	175 000	42	Activated sludge	Cl2
	Q3	900 000	146	Biological filters	-
	Q4	7 000	1.3	Biological filters	Cl2
	Q5	8 100	1	Trickling filters	Cl2
SA	SA1	1 300 000	135	Activated sludge	Stabilisation lagoons
	SA2	5 000	0.96	Oxidation ditches	Cl2
	SA3	7 377	1.77	Activated sludge	Cl2/UV [d]
	SA4	3 300	0.76	Anaerobic/aerobic lagoons	-

a Based on effective population serviced; **b** ACT = Australian Capital Territory, SA = South Australia; **c** chlorination; **d** ultra-violet radiation

5.3.5 Sampling methodology

In general, the protocols suggested by the United States Geological Survey in the National Field Manual for the Collection of Water-Quality Data were adopted for quality assurance (USGS 1999). Guidelines included avoiding the use of personal care products, such as perfumes, cologne or insect repellent.

Brown glass sampling bottles were used for sample collection and were thoroughly washed, along with all other experimental glassware, with Pyroneg™ detergent (free of alklphenol ethoxylates), and rinsed three times with de-ionized water and 18.2 $M\Omega cm^{-1}$ (Milli-Q®) water followed by 3 x 25 mL rinses of HPLC grade acetone and methanol. Glassware was then baked at 350°C overnight. A more detailed description of the bottle preparation procedure is given in Appendix 1.

Samples were collected in brown glass bottles capped with polytetrafluoroethylene (PTFE) lids, with no head space; and stored at +4°C. Sample volumes were 1 L for each replicate with all environmental analyses.

Duplicate samples were collected over a 24 h period for each WWTP location. The samples from Queensland were collected at 9 am, 12 pm, 5 pm and 9 am the next morning and shipped to the Adelaide laboratory separately. All samples from a 24 h period were then pooled at the Adelaide laboratory. The water samples from SA were collected hourly using 24 h composite samplers, while water samples from ACT were taken as grab samples. The dairy samples were taken as single grab samples from just below the surface of the selected streams.

For samples designated for chemical analyses, the bottles were acidified to pH 3 at each time interval, with colour-coded labels used to differentiate acidified samples for the chemical analyses from the samples for the biological analyses (which did not require any prior manipulation). All samples were transported to the Adelaide laboratory on ice in an insulated polystyrene box.

5.3.6 Sample preparation

An overview of sample preparation is given in Figure 5.3. Samples were filtered using a Whatman® GF/A (1.6 μm pore size) glass microfibre filter, followed by a Whatman® GF/C (1.2 μm pore size) glass microfibre filter. Samples were stored at +4°C until solid phase extraction (SPE) was performed (usually within 24 h). Extraction blanks containing 18.2 MΩcm^{-1} water only were treated the same as the effluent samples.

Samples were concentrated using SPE cartridges (Supelco ENVI-C18, 6 cc, 1 g) were conditioned with 5 mL methanol (100%) and 5 mL 18.2 MΩcm^{-1} water. The sample was loaded into the cartridge through PTFE tubing at a flow rate of approximately 3 mLmin^{-1}. The SPE cartridges were washed with 5 % methanol / 18.2 MΩcm^{-1} water (2 x 2.5 mL) before the sample was dried under vacuum. Samples were eluted with methanol and samples were reduced to dryness under a gentle stream of N_2. Samples were resuspended in 1 mL of methanol and vortexed for 30 s and transferred to 2 mL HPLC vials (with PTFE septa) and stored at –18°C until analysis.

Figure 5.2
Locations of sampling sites in rural areas.

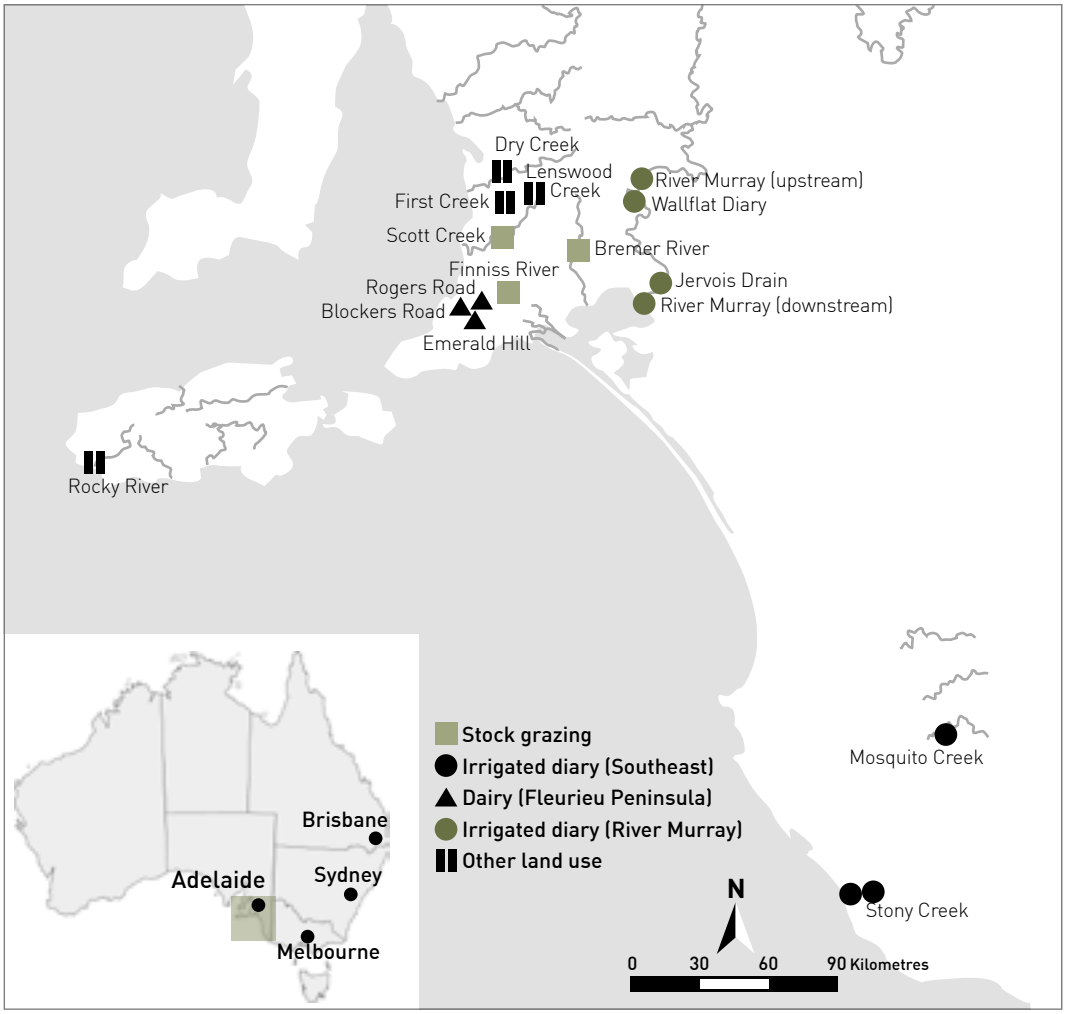
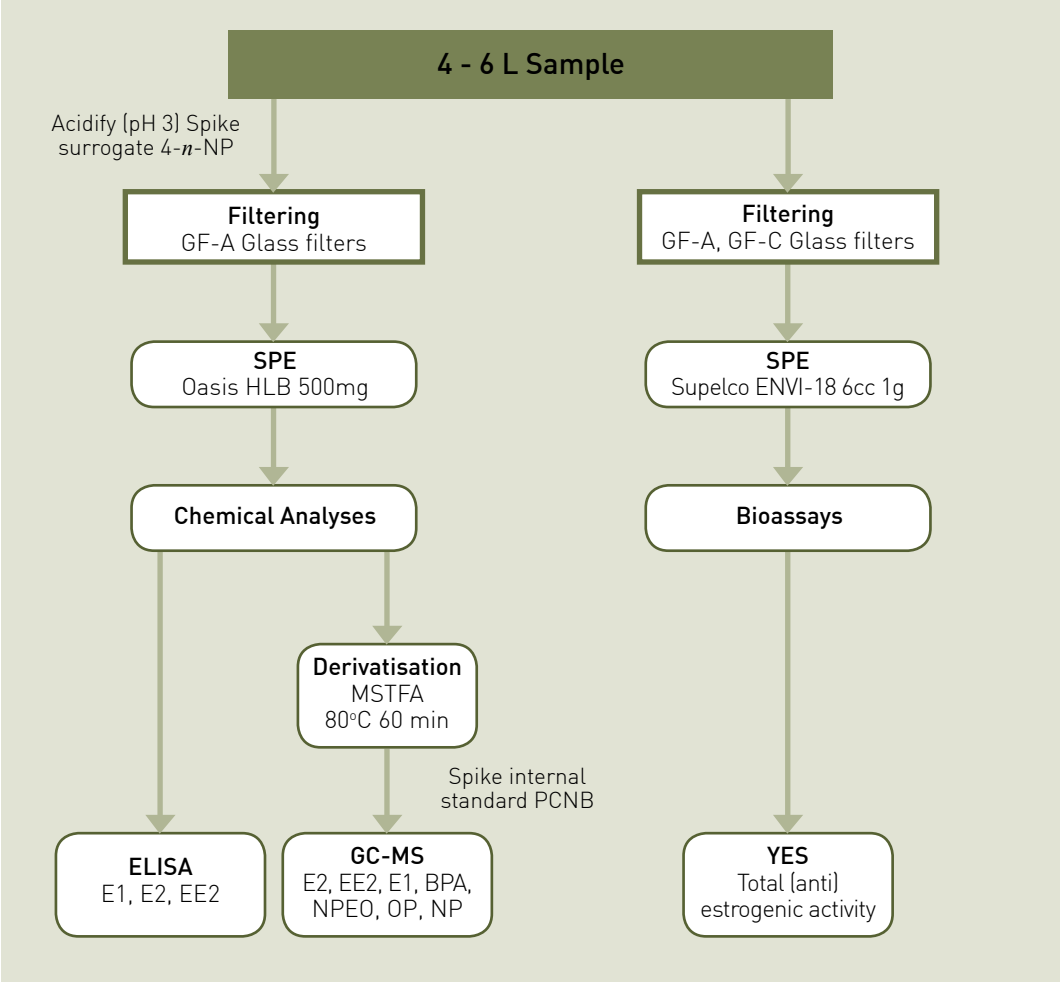


Figure 5.3
Overview of the approaches undertaken for preparation of environmental samples



5.3.7 Quality Assurance/Quality Control considerations

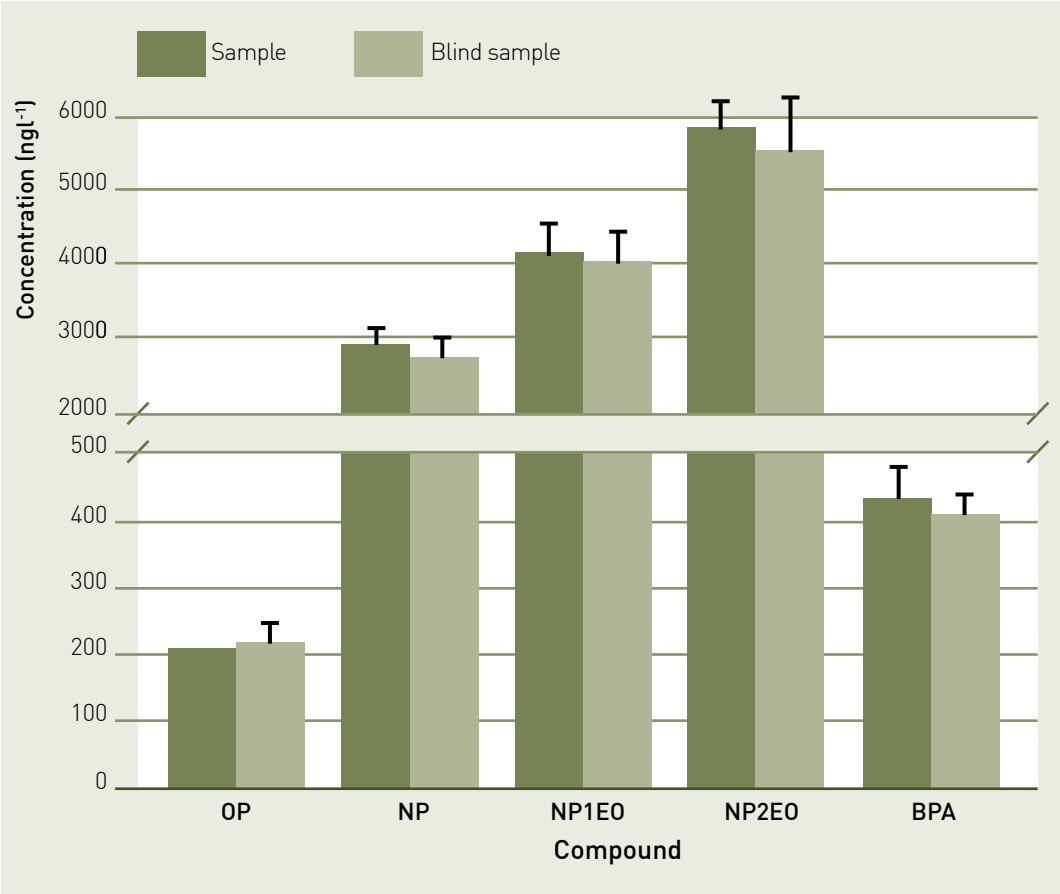
Field blank and blind samples were included in the sampling regime by the collaborating agency collecting the samples (Ecowise in ACT and Queensland EPA in Queensland).

Duplicate field blank samples were included during each sampling event. Two bottles filled with 18.2 MΩcm^{-1} water were included during field sampling, to check potential contamination from bottles and other sources. All samples were discarded if the concentrations of the alkylphenols, particularly NP, in the blanks were at significantly higher concentrations than the method limits of quantification. An overview of the concentrations of alkylphenols detected in the blank samples is given in Appendix 1.

Duplicate blind samples were collected, along with duplicate samples, from sites selected by the collecting agency, unknown to the analytical laboratory. These sites were then revealed following sample analysis, with the duplicate blind samples compared with the duplicate samples. An example is given in Figure 5.4 along with a summary in Appendix 1.

For samples undergoing GC-MS analysis, 4-*n*-NP was spiked to collected water samples prior to filtration and extraction to account for consistency of the extraction procedure (Figure 5.3).

Figure 5.4
Example of mean concentrations of xenoestrogens measured using GC-MS, comparing a blind sample with a labelled sample collected from an ACT WWTP. Error bars are standard deviations of the mean.



5.4 Analytical methods

5.4.1 Gas chromatography - mass spectrometry (GC-MS)

Gas chromatography - mass spectrometry (GC-MS) was used for the analysis of the xenoestrogens for the environmental sampling and fate studies. Analysis of the steroidal estrogens by GC-MS was only undertaken for the fate studies, where the samples were above the limit of quantification (Table 5.4).

Due to the low levels of these compounds that occur in the environment, clean-up and pre-concentration of water samples using solid phase extraction (SPE) was necessary for quantitative determination of the target analytes (Figure 5.3). Following SPE of the samples, the internal standard pentachloronitrobenzene (PCNB) was spiked into the samples and the analytes were derivatised by converting them into their trimethylsilyl (TMS) esters using *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) by heating at 80°C for 60 min. The lowest detection limits for the method (including the pre-concentration step) of the selected compounds by GC-MS were 5 ngL^{-1}. However, following spiking of environmental water samples, the limits of quantification (LOQ) for reporting the analytical method were set to 10 ngL^{-1} for BPA and OP, while E1, E2, EE2 and NP were set at 20 ngL^{-1}.

An Agilent 6890 GC, coupled with a 5973 MS, was used for GC-MS analyses. Separation was undertaken with a HP-5MS capillary column (30 m x 0.25 mm ID, film thickness 0.25 μm) and helium was used as the carrier gas (with flow rate of 1 $mLmin^{-1}$). A 1 μL aliquot of sample was injected in a splitless injection mode. The oven temperature was typically programmed as follows: 75°C (1 min) to 150°C ($10°Cmin^{-1}$) and then to 280°C ($15°Cmin^{-1}$) and held for 10 min. The injector and interface temperatures were set at 280°C, with the MS quad set at 150°C and the MS source at 230°C. The mass spectrometer was operated in the positive ion electron impact mode with an ionisation voltage of 70 eV using selected ion monitoring (SIM).

5.4.2 High pressure liquid chromatography (HPLC)

Analysis of E2, EE2, OP, NP and BPA was undertaken by HPLC using a fluorescence detector for assessment of their fate within aquifer sediments. This method has been described in detail elsewhere (Ying *et al*. 2003) but a brief description of this technique follows.

A Varian HPLC consisting of an autosampler (Model 9100), pump (Model 9012), fluorescence detector (Model 9070), on-line sample preparation system (Prospekt Model 9200) which was equipped with an SGE 250 GL4-ODS-H-12/5 column (250mm x 4mm ID). A 50 mL aliquot of sample was loaded onto the SPE cartridges, eluted with a 10 % methanol solution in water.

Analytes were quantified by fluorescence using an excitation wavelength of 230 nm and emission wavelength of 290 nm. Optimum separation on the column was achieved with a flow rate of 1 $mLmin^{-1}$ using a mobile phase gradient containing acetonitrile and water with the following gradient programme: 0-5 min 30-40 % acetonitrile, 5-10 min 40-60 % acetonitrile, 10-20 min 80 % acetonitrile (held for 5 min), followed by an isocratic purge for 5 min of 30 % acetonitrile, to give a total run time of 30 min. Following SPE, limits of quantitation were 10 ngL^{-1} for E2, EE2 and BPA and 30 ngL^{-1} for OP and NP.

5.4.3 Enzyme linked immuno-sorbent assay (ELISA)

As previously mentioned, the lowest method LOQs for the selected estrogens by GC-MS were 5 ngL^{-1}, although some previous studies have not been able to quantify the low levels of estrogens in some Australian samples (Leusch *et al*. 2005; Tan *et al*. 2007). For the present study we employed ELISA to address the potential for steroidal estrogens to be below the LOQ for GC-MS.

The ELISA is a rapid and sensitive technique used to quantify specific compounds within a sample. Recent work has indicated ELISA has similar sensitivity in a variety of environmental matrices, including wastewater, compared with HPLC coupled with triple quadrupole mass spectrometry (HPLC-MS/MS) and ultra-high pressure liquid chromatography coupled with time-of-flight mass spectrometry (UPLC-TOF-MS) (Farre et al. 2006; 2007). These chromatographic techniques are considered to be the best available techniques for environmental analysis of steroidal estrogens (Stuer-Lauridsen et al. 2005).

ELISA is based on the interaction of antigens and antibodies. Polyclonal or monoclonal antibodies (that have been raised against a particular analyte of interest) are used to detect the target protein in a sample by specific antigen : antibody interactions. In this project, ELISA kits (Japan Environ Chemicals Ltd) were used to determine the concentrations of

E1, E2 and EE2 present in the effluent samples, where their limits of quantification were below those for GC-MS. For complex environmental samples (such as WWTP effluent), a limited number of commercially available ELISA kits for quantifying steroidal hormones are available. The ELISAs were carried out according to the manufacturer's instructions, following filtration and pre-concentration with SPE. Appendix 3 gives a brief description of the ELISA methodology, standard curves and example calculations.

5.4.4 YES bioassays

In vitro assays have been commonly used to measure the total estrogenic and androgenic activities of environmental samples. For this project, a recombinant yeast screen, YES, was used for the detection of estrogenic activity. The ER-CALUX assay was undertaken at the NSW DPI, using the same sample extracts as were used for the YES assays.

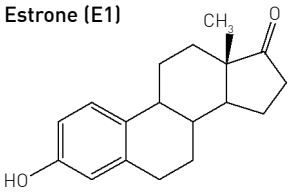
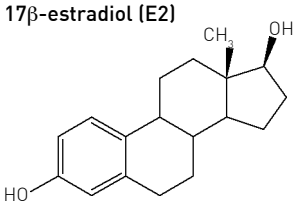
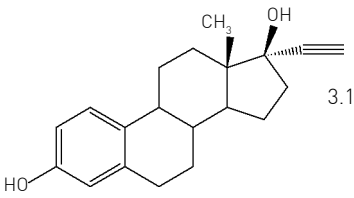
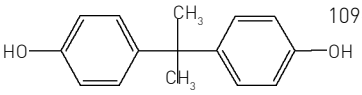
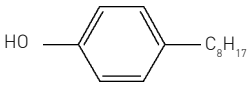
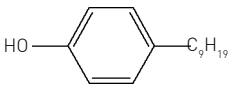
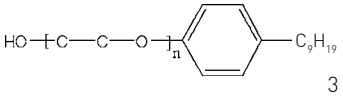
The YES assay has been previously described by Routledge and Sumpter (1996) and is now in use world wide and the assay was performed following this method. A more detailed description of the YES assay is outlined in Appendix 4.

Estradiol equivalents (EEqs) of the samples were determined by relating the response of the sample relative to the E2 standard curve. The relative potency, or estrogenic equivalency factor (EEF), was calculated from the ratio of the EC_{50} for the chemical versus the EC_{50} of E2, based on in vitro assays. The EEq of an estrogenic mixture was calculated as the sum of individual chemical concentrations (C_i) multiplied by their respective EEF:

$$EEq = \sum \{C_i x EEF\}$$

The relative potencies for each compound included in the EEqs are listed in Appendix 5 and were based on values derived from YES (Woods 2007).

Table 5.4
Physicochemical properties, analytical methodology and associated limits of quantitation of selected EDCs

Analyte	S[a] (mgL^{-1})	Log K_{ow} [b]	Analytical Methodology	LOQ [c] (ngL^{-1})	Comment
Estrone (E1) 	0.8-12.4 [d-g]	3.43 [f]	HPLC	10	Sorption experiments
			GCMS	5	Degradation study
			ELISA	0.05	Environmental survey
17β-estradiol (E2) 	3.1-12.96 [d-g]	3.94 [f]	HPLC	10	Sorption experiments
			GCMS	5	Degradation study
			ELISA	0.05	Environmental survey
17α- ethinylestradiol (EE2) 	3.1-19.1 [d-g]	4.15 [f]	HPLC	10	Sorption experiments
			GCMS	5	Degradation study
			ELISA	0.05	Environmental survey
Bisphenol A (BPA) 	109-300 [h-j]	2.20 [j]	GCMS	10	Sorption experiments Degradation study & Environmental survey
4-*t*-octylphenol (OP) 	12 [k]	4.12 [l]	HPLC	30	Sorption experiments
			GCMS	10	Degradation study & Environmental survey
4-nonylphenol (NP) 	7 [k]	5.76 [l]	HPLC	30	Sorption experiments
			GCMS	20	Degradation study & Environmental survey
NP-ethoxylates (NP_nEO) 	3 [k]	4.2 [l]	GCMS	20	Degradation study & Environmental survey

a water solubility, **b** octanol-water partition coefficient, **c** limit of quantitation, **d** Hurwitz and Liu 1977, **e** Yalkowsky 1999, **f** Yu *et al.* 2004, **g** Tabak *et al.* 1981, **h** Staples *et al.* 1998, **i** Hanselman *et al.* 2003, **j** Dorn *et al.* 1987, **k** Ahel and Giger 1993a, **l** Ahel and Giger 1993b.

6 Results and discussion

6.1 Sorption and degradation of EDCs in aquatic and terrestrial environments

6.1.1 Sorption

The sorption coefficients (both K_d and K_f) of E1, E2, EE2, BPA, OP and NP ranged from 2 to 2500 Lkg^{-1}, indicating a large variation in their affinities to the selected soil and sediment. BPA and E2 consistently had the lowest sorption coefficients, while NP had the highest affinity to the solid phase. Of the steroidal estrogens, EE2 had the highest affinity to the solid phase.

K_d values were found to increase with increasing organic carbon content of the selected solid phases. Therefore, the organic carbon normalised sorption coefficient (K_{oc}) was determined for each compound based on the following relationship:

$$K_{oc} = \frac{K_d}{f_{oc}}$$

where f_{oc} is the fraction of organic carbon in the solid phase.

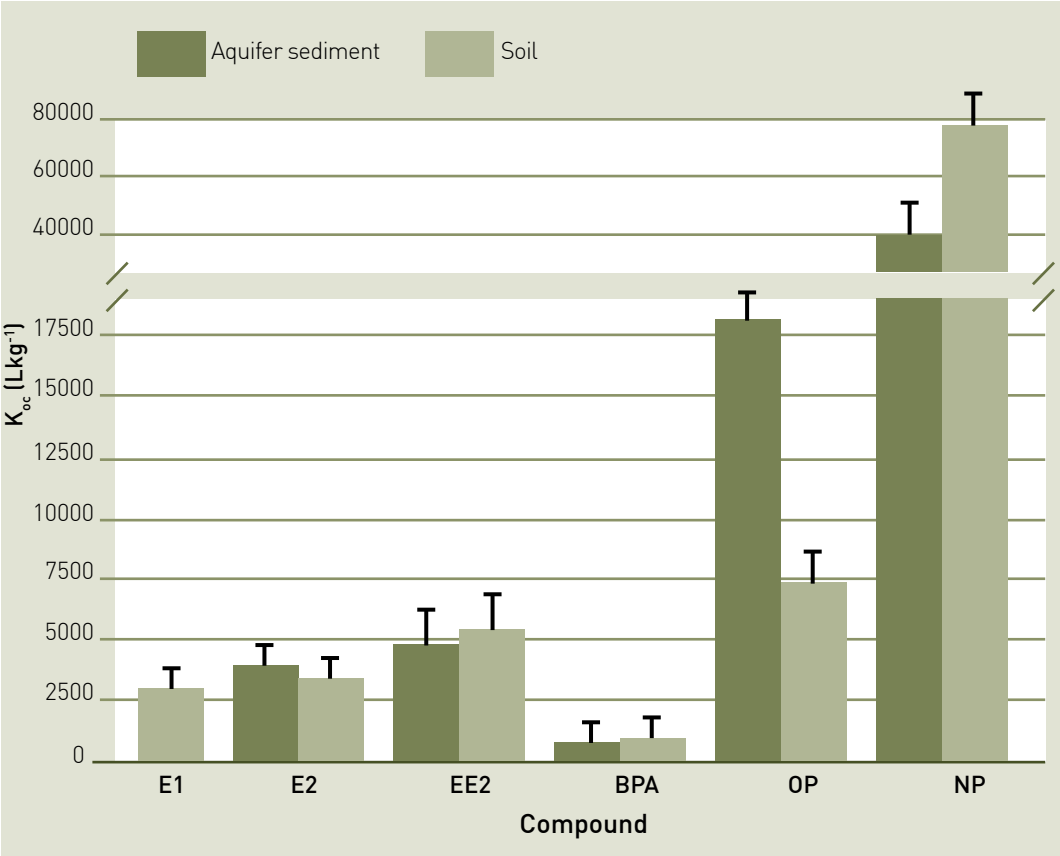
The K_{oc} values for the xenoestrogens ranged from 251 Lkg^{-1} for BPA in RF soil (TOC = 0.85 %) to 84,868 Lkg^{-1} for NP in WC soil (TOC = 2.9 %) (Figure 6.1). The K_{oc} for OP was substantially lower in the soils, compared with the sediment, although the mean value of 7405±672 Lkg^{-1} was still slightly higher than for steroidal estrogens. The K_{oc} of NP in the soils was higher than that in the sediment, although both K_{oc} values were significantly higher than all other compounds (Figure 6.1).

Previous studies have also reported high K_{oc} values for octylphenols and nonylphenols, indicating these xenoestrogens are likely to have a high affinity to sediments when they are released into aquatic ecosystems (Johnson *et al*. 1998; Sekela *et al*. 1999; Ferguson *et al*. 2001; Ying *et al*. 2003). Conversely, the xenoestrogen BPA was found to have a relatively low affinity for sorption, based on K_{oc} values from this and other studies (Staples *et al*. 1998; Sun *et al*. 2005).

The sorption of the steroidal estrogens were relatively moderate and were comparable with K_{oc} values in sediment and soils from previous studies ranging from 2961±629 Lkg^{-1} for E1 and 5432±1477 Lkg^{-1} for EE2 (Lai *et al*. 2000; Lee *et al*.2003; Ternes *et al*. 2004; Andersen *et al*. 2005). While these values are not as high as was found for the

alkylphenols, the sorption values for the steroidal estrogens indicate that sorption to solids in the aquatic environment is likely to be an important factor in their fate. The K_{oc} reduced the variability of the sorption coefficients for respective compounds, which indicated that organic carbon within the solid phase may have played an important role in the sorption of these compounds (Table 5.1).

Figure 6.1
Organic carbon normalised partition coefficient (K_{OC}) of selected EDCs with aquifer sediment and soil. The K_{OC} value for soils is the mean value for all soils. Error bars represent standard deviation of the mean.



6.1.2 Degradation

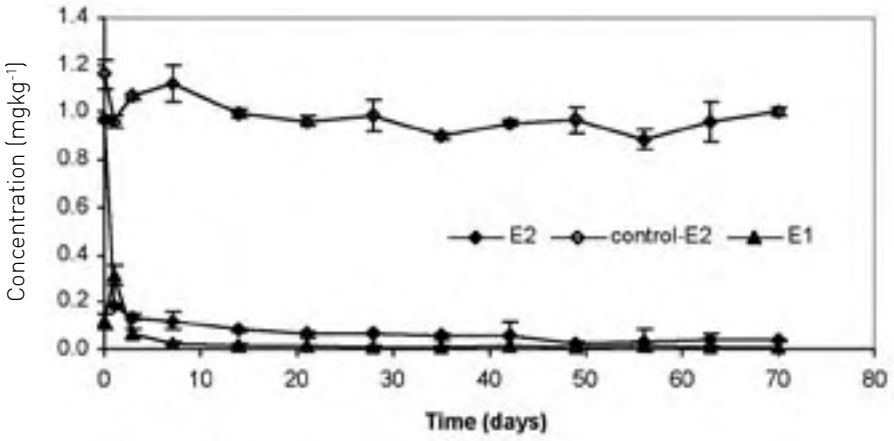
In biologically active soils under aerobic conditions, all of the EDCs showed a rapid and complete degradation (Figure 6.2). In sterilized control soils none of the compounds showed any appreciable degradation during the study period. This demonstrated that biological degradation was the main pathway of the transformation / degradation of the selected EDCs. Rapid degradation under aerobic conditions has also been previously found for the steroidal estrogens, although EE2 has the potential to be more resistant to degradation than the other steroidal estrogens (Ternes *et al*. 1999b; Layton *et al*. 2000; Jurgens *et al*. 2002; Lucas and Jones 2006). Also, based on these studies and the present one, the degradation rates of the steroidal estrogens are likely to be influenced by a number of environmental factors. The xenoestrogens have previously been found to rapidly degrade under aerobic conditions with environmental factors, such as temperature also likely to affect the rate of degradation (Staples *et al*. 1998; Manzano *et al*. 1999; Banat *et al*. 2000; Topp and Starratt 2000; Ying *et al*. 2002b; Shibata *et al*. 2006).

The degradation of the selected compounds was found to be negligible under anaerobic conditions in the test soil, sediment and aquifer sediment over the 70 d period (Figure 6.3). The exception to this was E2 which had a $t_{1/2}$ of 24 d. A comparison of the E2 dissipation behaviour under aerobic and anaerobic conditions showed that under anaerobic conditions not only is E2 degradation slower than under aerobic conditions but its breakdown product E1 also shows much greater persistence and possible accumulation. Other studies have also demonstrated that little degradation of steroidal estrogens and xenoestrogens is observed under anaerobic conditions (Ejlertsson *et al*.1999; Czajka and Londry 2006; Shibata *et al*. 2006).

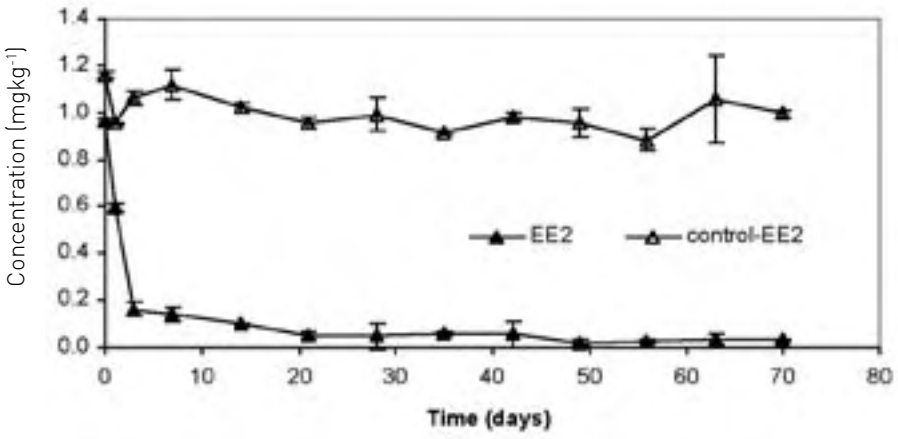
For all of the EDCs studied here the anaerobic conditions were found to be unfavourable for their rapid loss from the aquatic system. This finding may have an important bearing on the behaviour of EDCs in anaerobic sediments. The degradation rate of these compounds was found to be very slow in aquifer sediments devoid of significant biological activity even under aerobic conditions and while E2 and NP degraded, their degradation rates were much slower compared with the biologically active soils (Figure 6.4).

Figure 6.2
Aerobic degradation of selected EDCs in soil RF over 70 d. Error bars are standard deviation of the mean (from Ying and Kookana 2005 with permission).

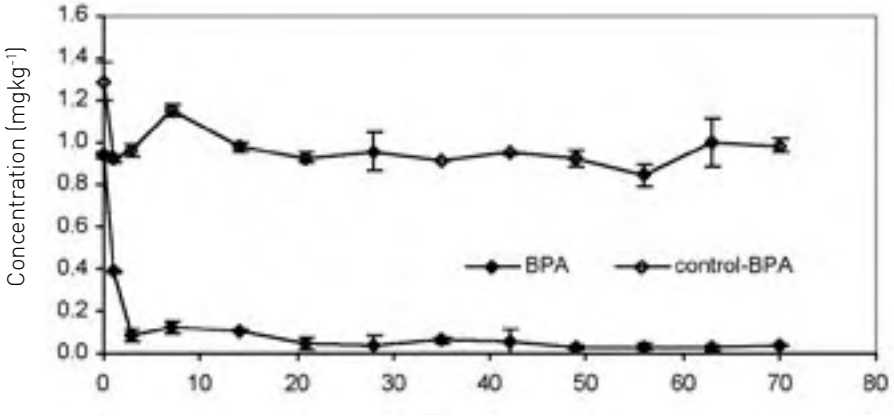
(A) 17β-estradiol (E2)



(B) 17α-ethynylestradiol (EE2)



(C) Bisphenol A (BPA)



(D) 4-*t*-octylphenol (OP), 4-nonylphenol (NP)

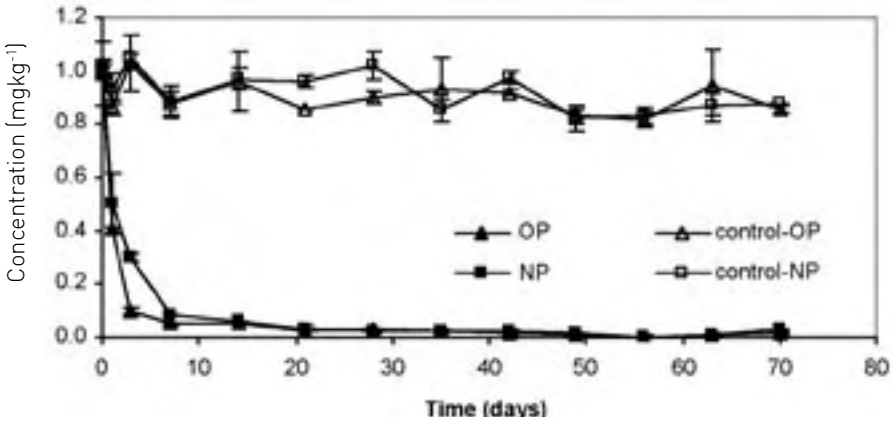
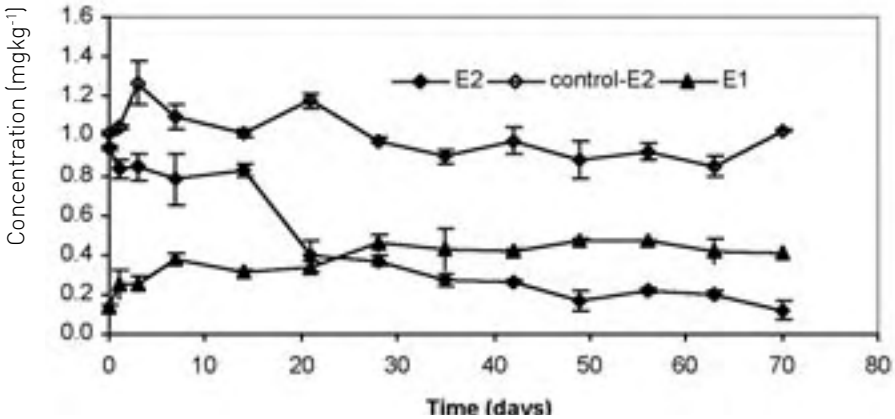
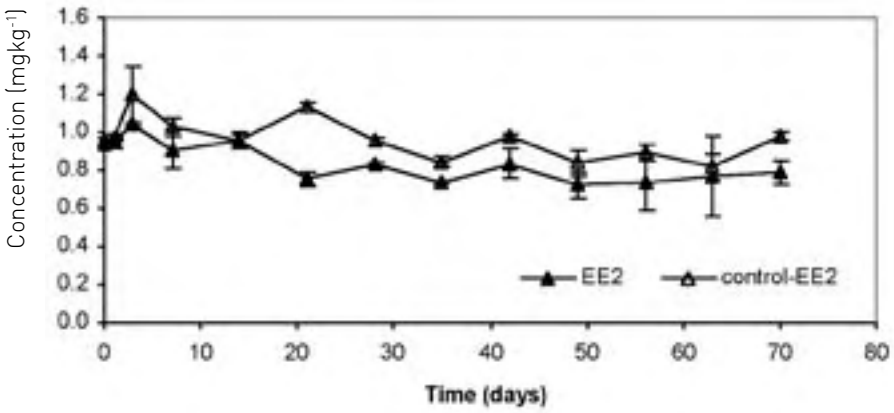


Figure 6.3
Anaerobic degradation of selected EDCs in soil RF over 70 d. Error bars are standard deviation of the mean (from Ying and Kookana 2005 with permission).

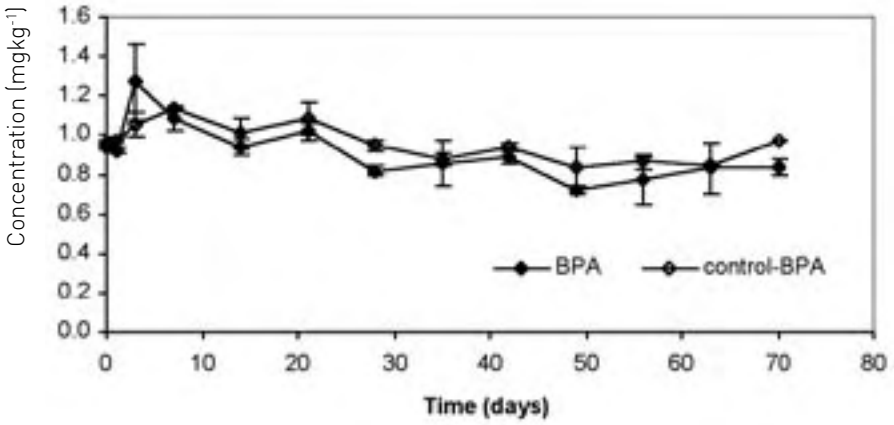
(A) 17β-estradiol (E2)



(B) 17α-ethynylestradiol (EE2)



(C) Bisphenol A (BPA)



(D) 4-*t*-octylphenol (OP), 4-nonylphenol (NP)

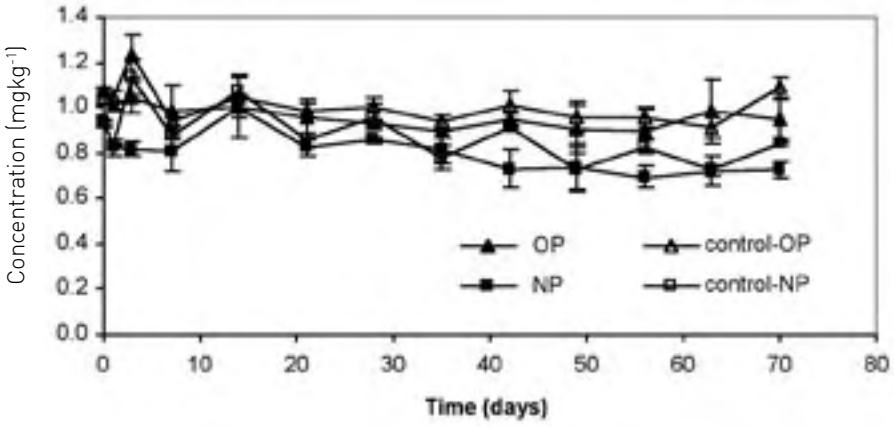
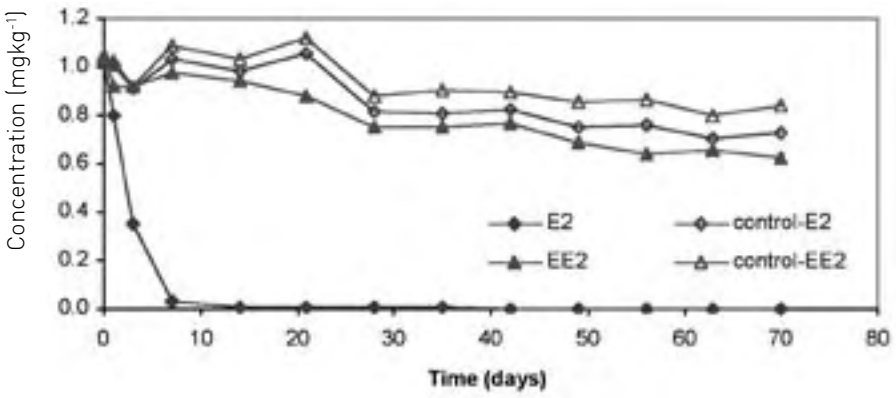
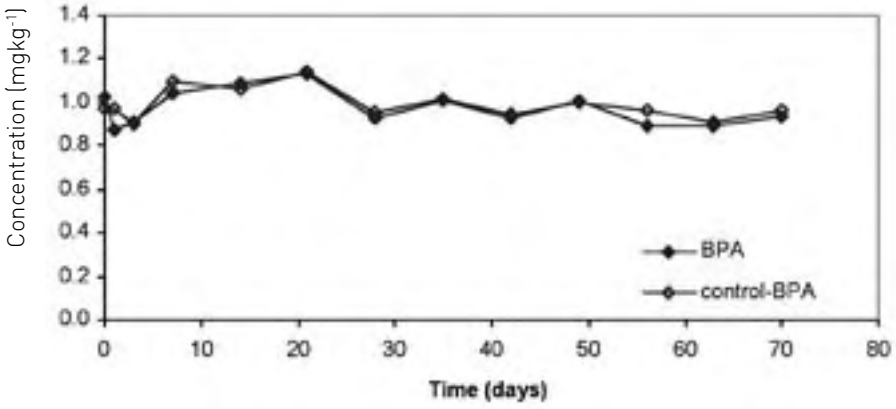


Figure 6.4
Aerobic degradation of EDCs in aquifer sediment and water over 70 d (from Ying *et al*. 2003 with permission).

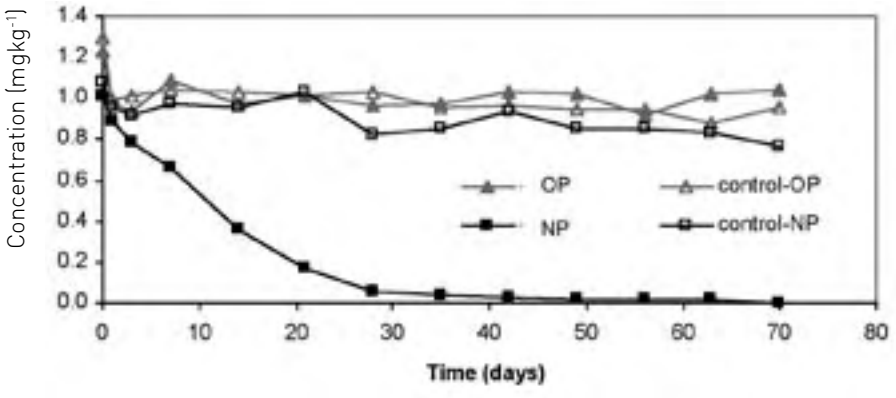
(A) 17β-estradiol (E2), 17α-ethynylestradiol (EE2)



(B) Bisphenol A (BPA)



(C) 4-*t*-octylphenol (OP), 4-nonylphenol (NP)



6.2 EDCs in sewage treatment effluents and the peri-urban environment

The concentrations of EDCs reported here must be considered within the context of the sampling methodology. That is, the reported concentrations are based on a snapshot of the levels of EDCs at a particular sampling time. In this case, a number of variables cannot be taken into account, including temporal variability in levels of wastewater, levels of contaminants input from domestic and industrial sources and external environmental factors that can influence the efficiency of the treatment process. Therefore, while the information presented an important baseline for the level of EDCs in WWTPs, the values should be regarded as indicative rather than definitive.

6.2.1 Influence of treatment plant technology on the concentration of EDCs in WWTPs effluent

In this work, the effect of treatment plant technology was tested in winter season (July 2005) in South Australia and therefore may represent the lower end of plant efficiency due to ambient conditions being unfavourable to biodegradation, which is an important mode of their removal through breakdown. The treatment technology was noted to have an important influence on the removal rates of the selected compounds from effluent (Figure 6.5 and Table 6.1). With respect to the removal of xenoestrogens, the SA1 WWTP (with tertiary treatment) was found to consistently remove the alkylphenol ethoxylates, alkylphenols and BPA at rates › 90 %. The removal rates were equally effective for SA2, SA3 and SA4 for OP and SA2 and SA3 for BPA. Removal of BPA was particularly low for the SA4 WWTP. Removal of the xenoestrogens selected for this study has also been noted in other studies, where a number of different treatment processes were used (Clara *et al*.2005; Auriol *et al*. 2006; Tan *et al*. 2007).

For the steroidal hormone E2, the removal efficiency was reasonably consistent amongst the treatment processes (ranging from 47-68 %), possibly due to its susceptibility to rapid degradation as discussed above. In contrast the removal of EE2 from water was highly variable among the four treatment plants. SA1 was the most effective in the removal of EE2 (72 %), compared with 58 % for SA2, ‹ 1 % for SA3 and 25 % for SA4. The removal rate of E1 from the WWTPs was also variable, which is to be expected due to both production of and degradation processes for E1 occurring simultaneously. Other studies have similarly reported that removal of steroidal estrogens can also be highly variable, making it difficult to predict their removal efficiency based on the selected treatment process (D'Ascenzo *et al*. 2003; Clara et al. 2005; Auriol et al. 2006; Hashimoto et al. 2007; Johnson *et al*.2007; Tan *et al*.2007). While previous work has identified chlorination as being highly effective in removing steroidal estrogens (Hu et al. 2003; Lee *et al*. 2004), this was not supported by the data from SA2 and SA3. However, other studies have also found chlorination similarly ineffective in the removal of steroidal estrogens, which may be related to chlorine being converted to chloramine (Itoh *et al*. 2000; Snyder et al. 2003; Chen *et al*. 2007).

The removal efficiencies are the combined effects of both the degradation of the compound in solution during the treatment process, as well as the partitioning of the compounds from solution into the solid phase. The occurrence of the latter process would seem to be supported from the sorption study, especially for the alkylphenols and the steroidal estrogens. While the previous section relating to degradation would also

seem to support rapid degradation occurring under aerobic conditions following sorption, the relative importance of these two removal processes should be more clearly defined through future mass balance studies that include the solid phase.

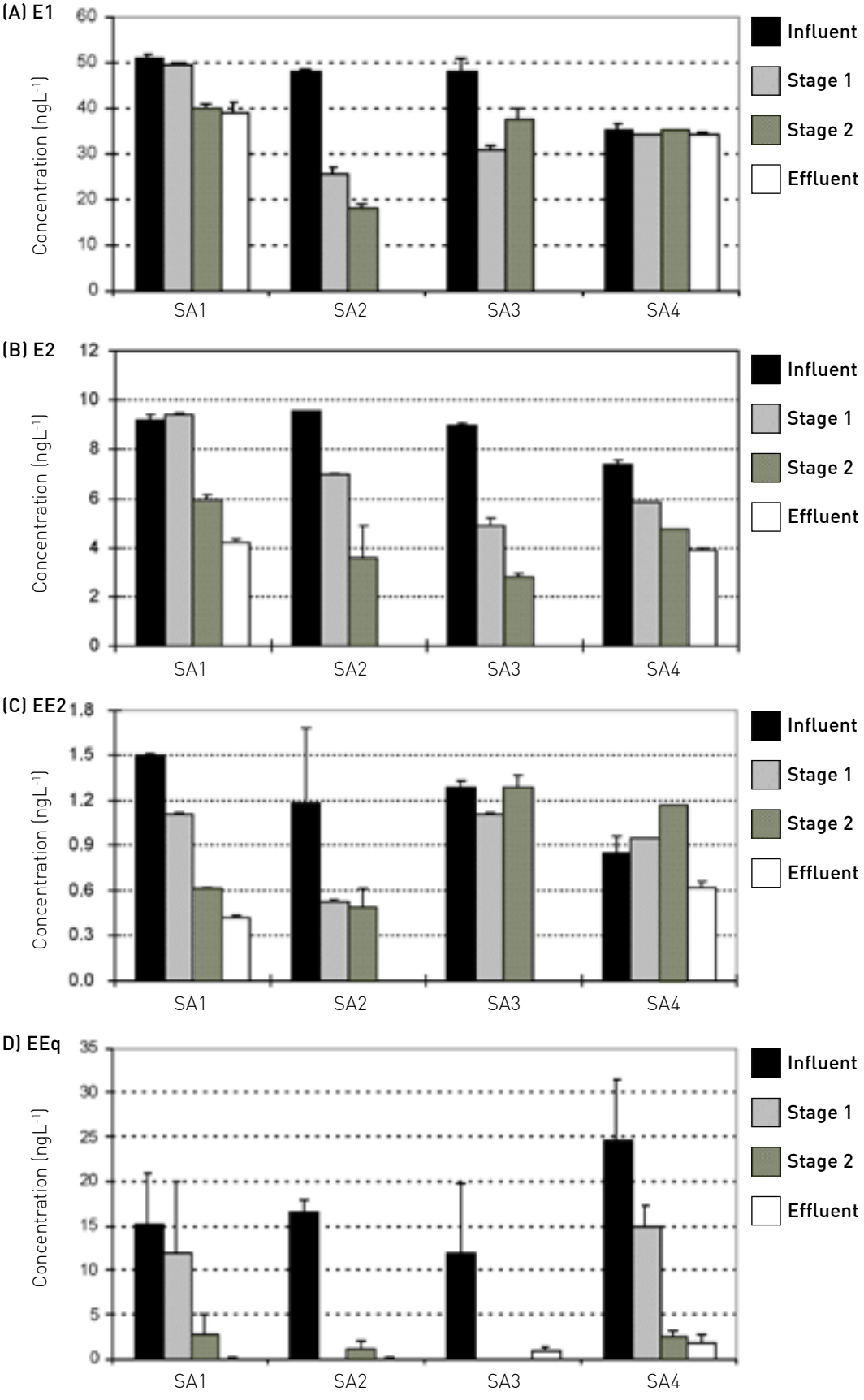
SA1, 2 and 3 WWTPs all had higher biological oxygen demand (BOD) of their influent relative to SA4 (Table 6.1). The suspended solids at these sites were also relatively higher than SA4, while the influent ammonia levels were relatively similar in all influents. The lack of correspondence of these parameters with removal efficiencies of EDCs is consistent with previous work showing that BOD and ammonia (NH_3) removal rates were not good predictors for the removal of steroidal estrogens (Johnson *et al*.2007).

Table 6.1
The removal efficiency in per cent of selected EDCs and WWTP water quality parameters at each South Australian WWTP.

Analyte	WWTP [a]			
	SA1	SA2	SA3	SA4
Estrone (E1)	24	63	22	0.9
17β-estradiol (E2)	54	62	68	47
17α-ethynylestradiol (EE2)	72	58	0.8	25
Bisphenol A (BPA)	92	96	92	20
4-*t*-octylphenol (OP)	98	96	93	90
4-nonylphenol (NP) [b]	92	80	70	64
Nonylphenol monoethoxylate & Nonylphenol diethoxylate (NP_nEO) [b]	92	80	70	64
17β-estradiol equivalents (EEq)	› 99	› 99	92	92
Biological oxygen demand (5-day)	› 99	› 99	› 99	93
Ammonia (NH_3)	› 99	96	93	36

a wastewater treatment plant; **b** values given are the sum of removal rates of NP, NP1EO and NP2EO

Figure 6.5
Measured concentrations of (A) E1, (B) E2, (C) EE2 and (D) EEq at defined stage of treatment process in SA1, SA2, SA3 and SA4 WWTPs. Each stage of the treatment process is another stage of treatment, from influent to final effluent, with samples taken in June 2005. Error bars represent standard deviation of the mean.



6.2.2 EE2: further considerations

One result that warrants further consideration is the concentrations of EE2 at SA2, SA3 and SA4 WWTPs. These WWTPs all service a relatively small population, compared with the SA1 WWTP and their relative flow rates are also considerably lower. A rough estimate of potential inflows of EE2 into the SA2, SA3 and SA4 was therefore calculated as follows (adapted from EMEA-CPMP 2005).

$$PC = \frac{E_{local}}{Wastew_{inhab} \; x \; Capacity_{WWTP}}$$

where PC is the predicted concentration in the WWTP, Elocal is the local emission to the WWTP (mgd^{-1}), $Wastew_{inhab}$ is the amount of wastewater per inhabitant per day (average 1.2 x 106 L person^{-1}d^{-1}) and $Capacity_{WWTP}$ is the capacity of the WWTP (average 5200 persons). E_{local} is calculated by:

$$E_{local} = \frac{Dose_{ai} \; x \; F_{pen} \; x \; Capacity_{WWTP}}{100}$$

where $Dose_{ai}$ is the maximum daily dose of the active substance (0.05 mg person^{-1}d^{-1}; DUSC 2004) and F_{pen} is the market penetration factor of the drug (%). In this case:

$$F_{pen} = \frac{Consumption_{year} \; x \; 100}{DDD \; x \; population \; x \; 365}$$

where $Consumption_{year}$ is the amount of active ingredient consumed (1 x 10^{7} mg year^{-1}; Braga *et al*. 2005), DDD is the defined daily dose of the active ingredient (0.05 mg 1000 persons^{-1} d^{-1}; estimated from highest daily dose used; DUSC 2004), population is the country's population (*ca*. 20 000 000; Australian Bureau of Statistics - www.censusdata.abs.gov.au) and 365 is the number of days in the year.

Based on these values and assuming no processes are involved in removing EE2 from the water column between consumption and the WWTP (e.g. metabolism / conjugation, sorption, degradation), concentrations would be expected to be around 6 ngL^{-1}. A similar analysis of SA1 WWTP would give an expected concentration of around double, or 13 ngL^{-1}. Raw sewage influent showed EE2 concentrations of 1.50±0.01 ngL^{-1} at SA1, 1.18 at SA2, 1.11±0.13 ngL^{-1} at SA3 and 0.85 ngL^{-1} at SA4. Factoring efficiency of removal between treatment technologies could make such values realistic, although previous findings on sorption and degradation rates may make these measured values at the higher end of the expected range. Considering the relative high *in vivo* estrogenic potency of EE2, the measured concentrations of EE2 in effluents would be expected to make an important contribution to the overall estrogenicity expressed in receiving waters. Therefore, further confirmation of these results using a selective analytical instrument, such as MS/MS, would be desirable for the assessment of EE2 concentrations in receiving waters.

6.2.3 Concentration of EDCs in WWTP effluents – influence of temporal and spatial factors

Concentrations of the selected EDCs in treated effluent from all 11 WWTPs from the three Australian regions sampled over two seasons, showed a fair degree of consistency, especially for the steroidal estrogens (Figure 6.6). The measured concentrations E2 ranged from 0.05-6.3 ngL^{-1} (median value 3.8 ngL^{-1}) and EE2 from 0.01-1.30 ngL^{-1} (median value 0.45 ngL^{-1}). In the case of E1 the range was 3.1-39.3 ngL^{-1} with a median concentration of 23.9 ngL^{-1}. Among the xenoestrogens, BPA and OP had the lowest concentrations with median concentrations of 21.5 and 39.5 ngL^{-1}, respectively. Considering their weak potency as endocrine disruptors, these levels are considered to be quite low. On the other hand, NP, NP1EO and NP2EO exhibited concentrations up to two orders of magnitude higher. For example, the concentration of NP ranged from 514 to 2991 ngL^{-1} with a median value of 1113 ngL^{-1}. Similarly, the parent compounds of NP were within the same order of magnitude with median concentrations of 1484 and 782 ngL^{-1} for NP1EO and NP2EO, respectively. These two compounds showed much greater sample to sample variations in their concentrations, possibly due to their propensity for transformation / biodegradation. Clearly, due to their higher loading in the effluents in Australia WWTPs, alkylphenols are of concern.

No clear seasonal effect on the levels of steroidal estrogens or xenoestrogens was noticeable and none of the WWTPs consistently showed higher levels of the EDCs compared with others.

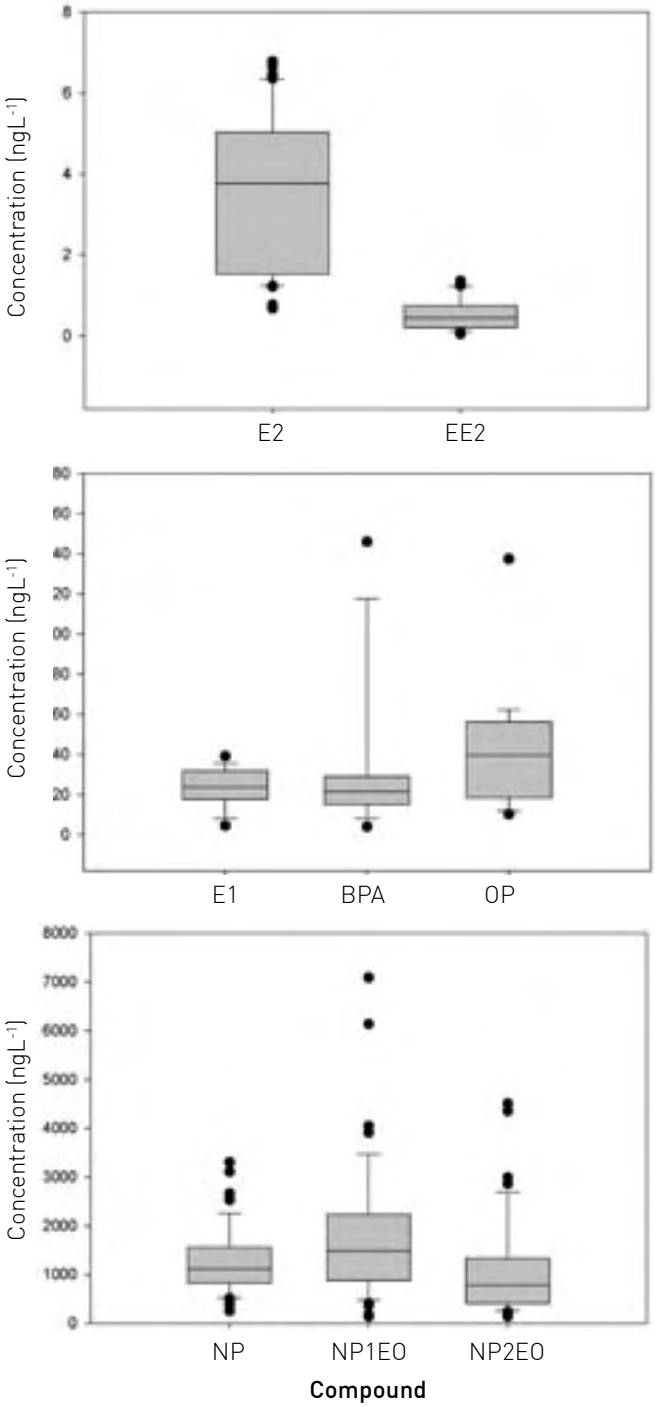
In summary, the surveys of the WWTPs at different periods of the year demonstrate a remarkable consistency between plants and sampling times with respect to absolute levels of respective EDCs. Furthermore, the relative levels between the EDCs at each WWTP, for each sampling period, were quite consistent. That is, NP1EO, NP2EO and NP were usually detected at high ngL^{-1} to low μgL^{-1} concentrations. The other phenolic xenoestrogens, BPA and OP, were commonly detected at low to mid ngL^{-1} concentrations. Of the steroidal estrogens, E1 was consistently detected at the highest levels, usually around an order of magnitude higher than E2. E2 was usually detected at low ngL^{-1} concentrations, while EE2 was detected from high pgL^{-1} to low ngL^{-1} levels, often around an order of magnitude lower than E2.

The levels of the steroid hormones in the effluent discharged in the environment were found to be inversely proportional to their potencies (Figures 6.6; see also Appendix 6). While E1 is a weaker estrogen than the other two, its concentration was an order of magnitude higher than E2. The same comparison can be made between E2 and EE2. Despite E1 having five times lower potency compared with E2, it may make a greater contribution to estrogenic load than E2, due to its higher concentrations. Among the xenoestrogens investigated only NP was at high enough concentrations to be an important contributor to total estrogenic load. The predicted EEq values ranged from 9 to 13 ngL^{-1} for SA, 3 to 13 ngL^{-1} for Queensland and 1 to 10 ngL^{-1} for the ACT samples. The overall median value for all 22 samples tested was found to be equivalent to 9 ngL^{-1} of E2.

Other studies in Australia have detected xenoestrogens (Tan *et al*. 2007) and steroidal estrogens at similar concentrations in effluents (Braga *et al*. 2005; Leusch *et al*. 2005; Ying *et al*. 2007), with xenoestrogens at mid to high ngL^{-1} concentrations and the steroidal estrogens were at low ngL^{-1} concentrations. These findings are also in line with a number of other international studies (Snyder *et al*. 1999; Ternes *et al*. 1999; Korner *et al*.. 2000;

Clara *et al*. 2005; Auriol *et al*. 2006; Hashimoto *et al*. 2007). It is therefore reasonable to conclude that the present study has contributed to the establishment of baseline levels of EDCs in Australian WWTPs, which are comparable with other countries. The results from study are within the wide range of concentrations of these compounds have been reported in overseas studies (Table 3.1), but, a true comparison with overseas studies is not possible here, as it can only be made when the analytical methodologies employed in different studies are the same.

Figure 6.6
Plots collating the measured concentrations of the selected EDCs in all WWTP effluents for both sampling periods (n=22). The lower and upper edge of the box represents the 25th and 75th percentile of samples, the lower and upper error bar limits represent the 10th and 90th percentile, while the bar across the body of the box represents the median value. Closed circles represent outliers either below the 10th or above the 90th percentile.



6.2.4 Estrogenicity of WWTP effluents – influence of treatment plant technology

The estrogenic activity within the WWTPs were found to be reduced by › 99 % for the SA1 and SA2 WWTPs, while 92 % of estrogenic activity was found to be removed by the SA3 and SA4 WWTPs (Table 6.1). These removal rates are comparable with the removal rate of OP and NP1EO and NP2EO, although the removal rates of the estrogenicity was considerably higher than the remaining xenoestrogens and steroidal estrogens.

The highest measured EEq value of 1.9±0.9 ngL^{-1} was in the SA4 WWTP effluent, ranging to the lowest value of 0.05±0.02 ngL^{-1} at the SA2 WWTP. This is in contrast with the predicted EEq values, based on the measured concentrations of the EDCs, which were considerably higher than the measured EEq values, which ranged from 8.1±0.4 ngL^{-1} at SA3 to 13.3±0.5 ngL^{-1} at SA1 (Figure 6.5).

Other studies in Australia (Leusch *et al*. 2005; Tan *et al*. 2007) and overseas (e.g. Korner *et al*. 2000; Svenson *et al*. 2003; Auriol *et al*. 2006) also reported similarly high removals of estrogenic activity following treatment in WWTPs, although this was often found to be highly variable and dependent on the type of treatment process employed. Furthermore, it was difficult to predict the efficacy of removal based on any single treatment process.

The relative concentrations and potency of the steroidal estrogens meant they contributed to › 99 % of the predicted estrogenic activity. However, their relative removal efficiency was considerably less and more variable than suggested by the measured estrogenic activity Also, the lower measured EEq values suggest anti-estrogenic activity within the effluent samples.

6.2.5 Estrogenicity of WWTP effluents – influence of temporal and spatial factors

In all cases, the estrogenicity measured using the YES assay was lower than the predicted EEq values based on the measured concentrations of the selected EDCs. Due to the relative potency and concentrations of the selected EDCs, the predicted EEq values were principally based on the measured concentrations of the steroidal estrogens. However, there was no significant relationship between the predicted and measured EEq values. Therefore, as with the previous section on treatment technology, the measured EEq was probably not only related to the concentrations of the steroidal estrogens in the samples but also with the anti-estrogenic compounds present. The primary mechanism of anti-estrogenic activity in chemically complex samples, including environmentally relevant water samples, is not yet known.

Previous research undertaken in southeast Queensland WWTPs found a more variable association between predicted and measured EEq values. In one study, Tan et al. (2007) reported that predicted EEq values from grab samples were considerably lower than measured EEq values, based on the E-screen bioassay. However, when a passive sampling device was used, the predicted EEq values were considerably higher than measured EEq values. Also, another study in Queensland reported the predicted EEq was greater than the measured EEq, based on E-screen, while an assay based on sheep uterine cells (ERBA) found predicted EEq values to be less than those measured from GC-MS (Leusch *et al*. 2005). These findings highlight the need for further research on the use of bioassays for assessing the estrogenic activity within environmental samples.

Furthermore, these findings, as well as the present work, demonstrate the need for combining *in vitro* assays and analytical chemistry for the assessment of EDCs in WWTPs. Significant advantages of *in vitro* assays over chemical analyses are that unknown components with estrogenic activity are not overlooked and that any combination effects are taken into account in the analysis. Chemical analysis of all compounds with potential estrogenic activity would be very costly and unknown estrogenic compounds, including metabolites, may still be present in environmental matrices. By a combination of the two types of approaches it is possible both to assess the estrogenic activity in a sample and to identify and quantify the compounds responsible for the estrogenic activity.

6.3 EDCs in rural streams

Using ELISA, E1 and E2 were detected in all samples taken from sites receiving inputs from dairy farming, stock grazing and horticulture. Low levels E1 and E2 were also detected in samples from sites selected as background sites, where no inputs of steroidal estrogens were expected (Table 6.2).

E1 concentrations ranged from 0.17 ngL^{-1} at National Park FC to 38.5 ngL^{-1} in dairy cattle effluent coming from a milking shed (Dairy WF). Concentrations of E1 were usually found to be higher than E2 at each sampling site, where E2 concentrations ranged from 0.5 ngL^{-1} at National Park FC to 8.6 ngL^{-1} also from Dairy WF. The concentrations found at the sampling sites could be expected based on the spatial sampling methodology. For example, the concentration of E1 and E2 at Dairy WF were the highest in the River Murray region, while the respective concentrations in the drain receiving the effluent from the dairy (Drain WF) were around half that of Dairy WF. Another drain servicing a dairy area has comparably lower concentrations of E1 and E2, while the most downstream site of the River Murray had returned to levels found at the most upstream location.

Higher concentrations of E1 and E2 were usually present in the aquatic systems receiving effluent from dairies, with areas influenced by urban inputs and stock grazing at slightly lower levels. Areas within horticultural areas had levels of E1 and E2 comparable with reference sites, although levels at National Park RR were notably higher.

Assessment of the estrogenic potential, based on ER-CALUX and YES assays, also indicated a similar pattern to the concentrations of E1 and E2 and the subsequent predicted EEq values. That is, the areas receiving inputs from dairy farms had the higher levels of estrogenic activity associated with them, with stock grazing also showing estrogenicity of effluents comparable with some dairy sites. However, the urban effluent exhibited relatively little estrogenic activity, for both ER-CALUX and YES assays, considering the concentrations of E1 and E2 that were detected at this site (Table 6.2).

The estrogenic activities detected by the bioassays were, in most cases, lower than the predicted EEq derived from the ELISA measurement of E1 and E2. The estrogenicity detected was often an order of magnitude lower than that predicted from the ELISA results. Conversely, at sites Dairy WF, Creek SC and Downstream SC there was reasonable agreement between the predicted EEq values and the bioassays. However, the relationship between the predicted EEq values and that observed with the bioassays was quite weak, although significant (r^2 = 0.26, p = 0.002). Another study by Vermiessen *et al*. (2005) found a stronger relationship (r^2 = 0.85) between measured and predicted EEq values. Unlike our study, Vermiessen *et al*. (2005) used similar dilutions of samples for chemical and bioassay analysis and noted a strong effect of dilution on the predicted EEq. This highlights the need of greater caution in reducing sample variability between bioassays and chemical assays.

In general, the ER-CALUX assay was more sensitive in detecting estrogenicity than the YES assay, although in some cases there was no difference found between the assays for estrogenic response (Figure 6.7). There was a reasonable and highly significant relationship between YES and ER-CALUX (r^2 = 0.63, p < 0.001). While both bioassays use the same reporter gene approach, the YES assay is often found to be less sensitive than mammalian cell-based assays, such as ER-CALUX. A number of reasons can be attributed to the lower sensitivity in YES, such as differences in uptake of compounds over cell membranes, different sensitivities of cellular transcription factors, the presence of endogenous yeast binding proteins and the difference in sensitivity of colourimetric (YES) and luminometer-based (ER-CALUX) analytical techniques (Zysk *et al*. 1995; Kralli *et al*. 1995; Halachmi *et al*. 1994; Feldman *et al*. 1982; Villeneuve *et al*. 1998).

The E1 and E2 concentrations in the drains and receiving rural riverine systems were similar to those detected in the WWTP effluents. In general, the concentrations of E1 were slightly lower in the rural systems, compared with the WWTP surveys, while the concentrations of E2 measured were more consistent between the rural and WWTP surveys. Therefore, the ratio of concentrations between E1 and E2 were lower during the surveys of rural drains and receiving systems, whereas there was more divergence between concentrations of E1 and E2 in WWTP effluents. For example, the highest ratio between the concentrations of E1 and E2 in the rural sampling was around the same as the lowest ratio for the WWTP effluent samples (*ca.* 5 : 1). The formation of E1 from the oxidation of E2 was indicated in the degradation study, as well as other studies (Lai *et al*. 2000; Colucci *et al*. 2001). It may therefore be worth further exploring the ratio of E1 : E2 in environmental samples as an indication of the extent of treatment these two steroids have undergone.

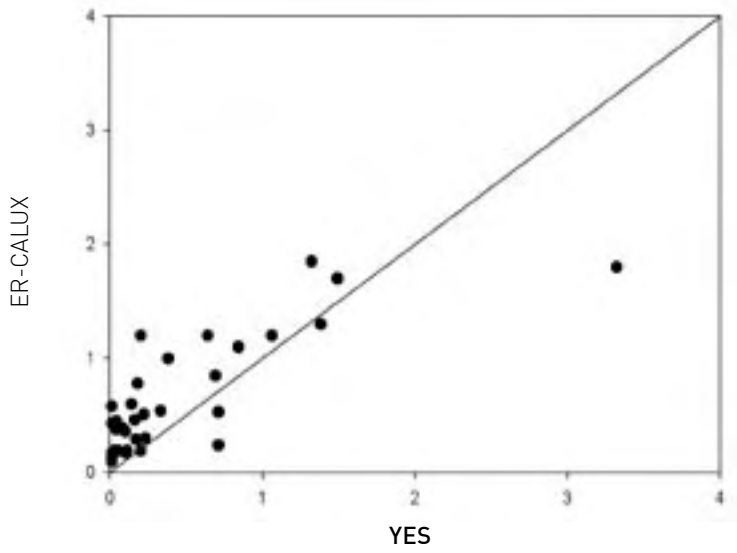
It is noteworthy that the concentrations at sites selected for background levels showed high pgL^{-1} to low ngL^{-1} concentrations of E1 and E2. Each of these sites was located in an area that was considered to be free of potential inputs and, therefore, assigned as reference areas. Estrogenicity, based on the bioassays, was also noted at these background sites. YES and ER-CALUX both confirmed estrogenicity was present in the water samples at the Upstream SC and Upstream RM sites, although only ER-CALUX detected estrogenicity at National Park RR, National Park FC and Upstream EH. The concentrations of E1 and E2 at the reference sites were relatively comparable between sites, although concentrations were around an order of magnitude lower at National Park FC. Explanations for the levels of steroidal estrogens at each of these sites are likely to be site-specific and indicate why selection of background sites can be problematic. For example, E1 and E2 in the Upstream RM sites may be due to human activity, such as recreational boating further upstream. Also, the presence of wild pigs was noted in the vicinity of the low-flow ponds around where the samples were collected for National Park RR. These pigs may have introduced estrogens into this area while sourcing water. Further work using a more selective analytical tool allowing more specific assessment of compounds in samples, as previously discussed for EE2, would be desirable for confirmation of these findings.

Table 6.2
Measured concentrations of E1, E2 and EEq (based on YES and ER-CALUX). Also, predicted EEq (based on combined potency and concentration of E1 and E2) is used as a comparison.

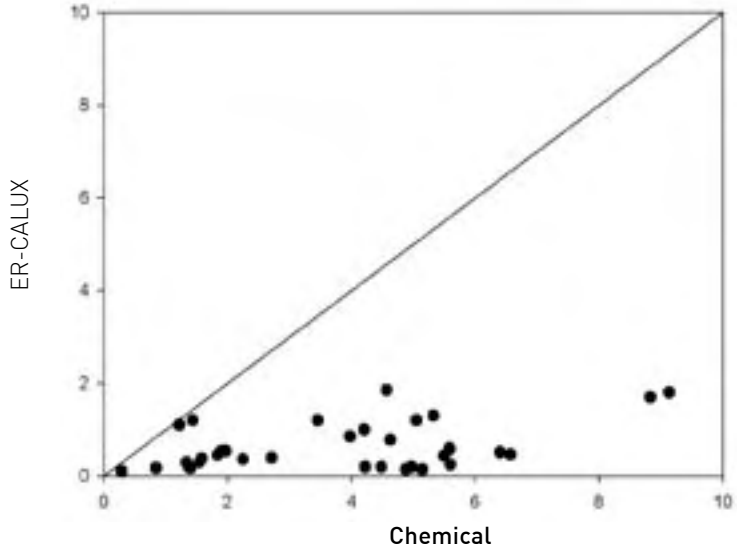
Land use and sites	E1 (ngL^{-1})	E2 (ngL^{-1})	Predicted EEq (ngL^{-1})	YES EEq (ngL^{-1})	ER-CALUX EEq (ngL^{-1})
Irrigated Dairy, South East					
Upstream SC (Stony Creek)	3.09 ± 0.14	1.00 ± 0.10	1.93 ± 0.06	0.52 ± 0.269	0.54 ± 0.01
Dairy input MC (Mosquito Creek)	13.79 ± 0.14	2.34 ± 0.06	6.48 ± 0.12	0.19 ± 0.042	0.49 ± 0.04
Downstream SC (Stony Creek)	5.19 ± 0.36	2.71 ± 0.31	4.27 ± 0.42	1.01 ± 0.44	1.35 ± 0.71
Dairy on Fleurieu Peninsula					
Upstream EH (Emerald Hill)	3.99 ± 0.37	3.81 ± 0.08	5.01 ± 0.19	‹ LOQ	0.14 ± 0.09
Dairy input BD (Blockers Road)	6.85 ± 0.35	3.13± 0.09	5.19 ± 0.19	1.22 ± 0.226	1.25 ± 0.071
Dairy input RR (Rogers Road)	7.47 ± 1.04	2.67 ± 0.66	4.91 ± 0.97	0.46 ± 0.361	0.22 ± 0.03
Irrigated Dairy, River Murray	2.24 ± 0.25	1.04 ± 0.11	1.71 ± 0.18	0.04 ± 0.01	0.42 ± 0.10
Upstream RM (River Murray)					
Drain WF (Wallflat Dairy)	18.71 ± 1.17	3.37 ± 0.57	8.98 ± 0.22	2.41 ± 1.29	1.75 ± 0.07
Dairy WF (Wallflat Dairy)	38.46 ± 0.42	8.61± 0.18	20.15 ± 0.05	13.56 ± 0.05	10.60 ± 6.22
Drain JD (Jervois Drain)	9.11± 1.33	2.37± 0.28	5.10 ± 0.68	0.16 ± 0.03	0.69 ± 0.13
Downstream RM (River Murray)	2.08± 0.21	0.81± 0.09	1.44 ± 0.15	0.20 ± 0.04	0.30 ± 0.01
Stock grazing					
Creek SC (Scott Creek)	1.57± 0.20	0.86 ± 0.09	1.33 ± 0.15	0.74 ± 0.141	1.15 ± 0.07
River FN (Finniss River)	5.75± 0.78	2.11 ± 0.30	3.83 ± 0.53	0.29 ± 0.127	1.10 ± 0.20
River BR (Bremer River)	3.56± 0.40	1.41 ± 0.21	2.48 ± 0.33	0.09 ± 0.014	0.38 ± 0.05
Other land-uses					
Horticulture LC (Lenswood Creek)	1.47± 0.22	0.94 ± 0.08	1.38 ± 0.17	0.11 ± 0.007	0.18 ± 0.14
Urban DC (Dry Creek)	6.08± 0.40	2.90± 0.22	4.73 ± 0.34	0.04 ± 0.014	0.19 ± 0.09
National Park FC (First Creek)	0.17± 0.15	0.52 ± 0.35	0.57 ± 0.39	‹ LOQ	0.13 ± 0.05
National Park RR (Rocky River)	4.23± 0.02	4.27 ± 0.06	5.54 ± 0.07	‹ LOQ	0.50 ± 0.10

Figure 6.7
1:1 plots showing the relationship between measured EEq values based on (A) YES and ER-CALUX, (B) predicted EEq (based on chemical concentrations) and ER-CALUX and (C) predicted EEq (based on chemical concentrations) values and YES.

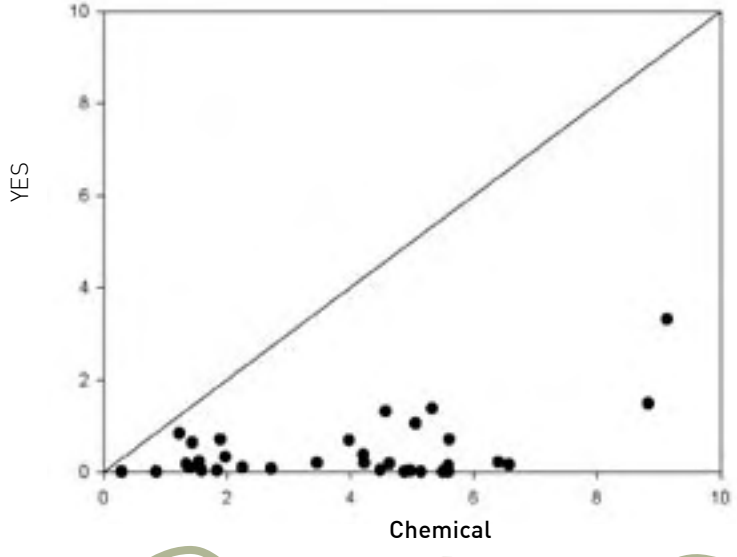
(A)



(B)



(C)



6.4 Anti-estrogenicity: a case study

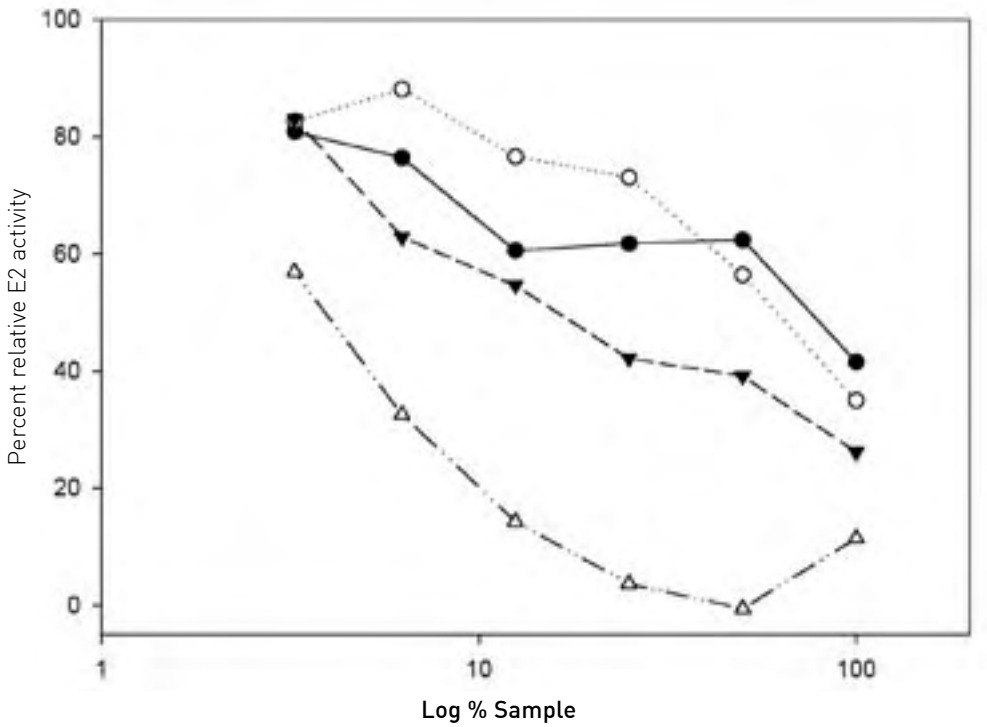
The YES assay and ER-CALUX generally showed a lower total estrogenic potential than predicted through the combined concentration of selected EDCs by chemical analysis and ELISA (Table 6.2; see also Executive Summary Figure 1 and Appendix 6). The measured EEq values were consistently lower (one to two orders of magnitude) than predicted EEq values, regardless of sampling period and the type of treatment technology employed. Based on these observations, it was suspected that there was some degree of anti-estrogenicity associated with the effluent samples.

The majority of samples exhibited varying degrees of anti-estrogenic activity when tested in the anti-estrogenic screen. The magnitude of anti-estrogenicity was not dependent on the spatial or temporal differences or the treatment plant technology employed. Figure 6.8 contains representative dose-response curves for anti-estrogenic activity for four effluents. The anti-estrogenic activity within the samples may account for the large differences between the observed and calculated EEqs. There are several possible mechanisms that may contribute to this activity including the bioavailability of the compounds to participate in ligand binding in the YES assay, matrix effects and presence of unknown estrogenic / anti-estrogenic compounds.

Figure 6.8
Response of YES assay to dilutions of four effluents, all spiked with (percent relative E2 activity) for

Effluent 1 S1 Effluent 1 S2
Effluent 2 Effluent 3 E2

was set at a sub-maximal level in all dilutions of samples and used as the basis of relative E2 activity (i.e. 100 % response).



The predicted EEq values were calculated on the basis of 10 estrogenic compounds, of which three contributed › 99 % of calculated EEq values. This does not take into account the presence of other unidentified compounds and their influence on the overall estrogenicity of the sample. The lower EEqs calculated with the YES assay may be due to compounds present in the extract that interfere with the YES assay or form complexes with dissolved organic material in the wastewater (Snyder *et al*. 2001). The concentration of hydrophobic organics on reverse-phase media prior to estrogenic or anti-estrogenic activity measurement may exacerbate such matrix effects. Several studies have reported the ability of E2 to interact with colloidal material in wastewater, which may render E2 or other estrogenic compounds unavailable to produce a biological effect (Williams *et al*. 1999; Bowman *et al*. 2002).

Suppression of the YES signal was observed in this study at lower dilutions of collected samples due to toxicity, with similar observations also reported by Vermeirssen *et al*. (2005). To overcome the issue of cell toxicity, the EEq of effluent samples were calculated at the lowest effluent dilution which fitted with the linear portion of the standard dose-response curve (*see* Appendix 4). The dilution was therefore likely to have reduced the effect of the matrix, with all samples generally parallel with the E2 standard curve.

Another possible mechanism is through biochemical pathways, whereby an antagonist competes with estrogens for hER binding sites (Katzenellenbogen *et al*. 1995). The difference between hER agonists and antagonists is primarily due to the ligand moiety or side chain which varies in length and orientation. A recent study by Conroy *et al*. (2005) suggested that weak estrogens may play an anti-estrogenic role when present with potent ligands, whereby the weak estrogenic compound interferes with the binding of the potent E2.

Calculated EEq values also rely on the compounds present in a mixture being additive in their effects. The EEq does not account for the presence of interactions and / or antagonism between chemicals within a sample (Korner *et al*. 2001). These effects may be due to unknown chemicals in a sample either enhancing or reducing the calculated EEqs and/or EEqs from *in-vitro* assays and is especially true for complex environmental samples such as WWTP effluents (Korner *et al*. 2001; Huggett *et al*. 2003).

The integrative nature of the bioassays used in this study suggests there was anti-estrogenic activity within the collected samples, which gave a reduced EEq value relative to the measured EEq values. It is evident that further investigation should be undertaken to determine whether this apparent anti-estrogenicity was actually due to the presence of antagonistic compounds. Therefore, based on the current evidence it seems prudent to classify the anti-estrogenicity observed as being related to mitigation of the estrogenic activity, rather than solely due to antagonism of the ER. Separating these two effects would be facilitated with *a priori* targeting of potential anti-estrogenic compounds, using chemical analytical techniques. Future work will also need to focus on measuring both predicted and measured EEq in the same sample at the sample dilution level to reduce variability in the data set.

7 Conclusions

7.1 Key findings and implications

7.1.1 EDCs of concern

Considering the complex nature of EDCs and growing research capacity this project is exploratory. Findings arising from the present work could provide a snapshot of levels in the Australian riverine environment and also form the basis of future research. A group of representative estrogenic compounds was selected based on the perceived risk from their presence in riverine systems. This assessment of EDCs indicated that the steroidal estrogens were likely to represent a risk in the aquatic environment based on their estrogenic potency and occurrence. The concentrations at which E1, E2 and EE2 have been detected in international surveys are at levels that are thought to be of significance from previous *in vitro* and *in vivo* assessment of endocrine disruption. The steroidal estrogens in particular are likely to contribute to estrogenicity in the aquatic environment. Relative to E2, EE2 is found at lower concentrations but has higher estrogenic potency. Similarly, E1 has lower estrogenic potency than E2 but is usually detected in the environment at higher concentrations. Therefore, despite the lower potency of E1, considering E2 alone could substantially underestimate the estrogenic load within a receiving system. Xenoestrogens selected for assessment have a considerably lower estrogenic potential than the steroidal estrogens, although their environmental concentrations in other international studies were high enough to warrant their investigation in this study. The xenoestrogens studied included nonylphenol ethoxylates (NP1EO and NP2EO), alkylphenol ethoxylate metabolites (OP and NP) and BPA.

In summary:

- EDCs selected for this study were selected based on the literature review and a preliminary assessment of potential risk to the aquatic environment due to their estrogenic potency and likely environmental concentrations;
- Potency and environmental concentrations are both important factors to take into account for overall hazard assessment. For example, EE2 is consistently found at the lowest concentrations of the steroidal estrogens but, due its high relative potency, the overall contribution of EE2 to estrogenic potential in aquatic systems is significant;
- Steroidal estrogens selected for this project included E1, E2 and EE2;
- Xenoestrogens selected for this project were the alkylphenol ethoxylates (NP1EO and NP2EO), alkylphenols (NP and OP) and BPA.

7.1.2 Environmental fate of EDCs – sorption and degradation

The sorption of the selected EDCs was highly variable, with K_{OC} values ranging from 250 to 85,000 Lkg^{-1}. The alkylphenols (OP and NP) adsorbed to the solid phase to a significant extent, with the steroidal estrogens adsorbed to a lesser extent and BPA had the lowest affinity. However, all compounds exhibited some extent of adsorption to solids and, therefore, it is likely that the sediment phase in aquatic environments would be an important compartment for EDCs. Sorption increased with increasing organic carbon content of the solid phase, indicating sorption would become a more important influence on environmental fate as the organic carbon content of the solid phase increased.

Degradation of the test compounds was due principally to biotic degradation processes, since all compounds were stable under abiotic conditions. In general, rapid degradation of the test compounds was observed under aerobic conditions within soils. However, a slower rate of aerobic degradation was observed for EE2, BPA and OP when they were spiked to sediment obtained from an aquifer, with limited microbial activity. All compounds were resistant to degradation processes under anaerobic conditions, although E2 was found to degrade under anaerobic conditions, with more than 80 % degrading after 70 d. However, the subsequently formed E1 was reasonably stable under anaerobic conditions, although the formation of E1 from E2 would lead to a general reduction of estrogenic potential due to the lower potency of E1.

Degradation of these compounds in the aquatic environment may therefore be expected to be reasonably rapid, unless the system is anaerobic or does not readily support a degrading microbial community. While microbial degradation is dependent on a diverse array of factors such as the microbial communities present, their access to nutrients and environmental factors, microbial communities are often associated with sediments in aquatic systems (Warren et al. 2003). Therefore, sorption to sediments may further support rapid degradation within a riverine system. However, this needs to be investigated in a test system that more closely reflects environmental conditions.

With respect to degradation patterns within WWTPs, the conditions under which a WWTP operates would be expected to be conducive to biotic degradation processes playing a significant role in removal of the EDCs. However, it is difficult to ascertain, from this study and others, which treatment technology would be the most efficient in removal of EDCs. Removal from the aqueous phase would also be related to adsorption to biosolids, which are sequentially removed during filtration and sedimentation.

In summary:

- All selected EDCs were found to associate with sediment, biosolids and soils;
- OP and NP were adsorbed to a greater extent than the steroidal estrogens (E1, E2 and EE2), while BPA adsorbed to the least extent;
- Biodegradation of the selected EDCs was rapid under aerobic conditions in soils;
- There was little degradation observed under anaerobic and sterile conditions or when aquifer sediments with limited biological activity were used;
- Sorption and biodegradation are both likely to play an important role in the fate of these EDCs in both WWTPs and the aquatic environment under conducive conditions.

7.1.3 EDCs in WWTPs - influence of treatment plant technology

Assessment of the technology employed in a WWTP indicated that the effectiveness of removal can vary significantly depending on the treatment technology. The examples of the South Australian WWTPs demonstrated tertiary treatment of wastewater, where active aeration of the influent was the most effective in removing the xenoestrogens. The SA2 (oxidation ditches) and SA3 (bioreactors and UV disinfection) WWTPs, also had reasonable removal rates for NP, NP1EO and NP2EO. The lagoon system at SA4 was the least efficient at removing the xenoestrogens.

The removal efficiency of the steroidal estrogens was consistently lower at all WWTPs, compared with the xenoestrogens, with removal rates for E1, E2 and EE2 ranging from ‹ 1 % to 72 %. The performance of the WWTPs was variable for each steroidal estrogen, although SA2 had the most consistent removal rates of the steroidal estrogens, with removal rates ranging from 58 % to 63 %. Conversely, the removal of estrogenic activity was found to be efficiently removed from all WWTPs, with 92 % removed from SA3 and SA4 WWTPs, while › 99 % was observed at SA1 and SA2 WWTPs. It was therefore difficult to define a WWTP technology from the surveyed WWTPs that would be the most effective in removing the selected EDCs, as has been observed from other research. The above findings are based on data from a single season. For a more reliable picture on treatment technology efficiency, more data over several seasons, including mass balance studies, are needed.

Overall, the removal of the total estrogenic load was found to be highly efficient at all plants, with all WWTPs removing › 92 % of estrogenicity. This finding highlights the need for taking both chemical and biological assays into account.

There is still ambiguity relating to the removal processes of EDCs in WWTPs. For example, the removal of EDCs may be related to either biodegradation or sorption to solids within the wastewater stream. While removal from wastewater is an important step in reducing loads of EDCs in effluents, application of biosolids to land from WWTPs may be simply moving these compounds to another environmental compartment.

In summary:

- The technology used within the WWTPs had an influence on the concentrations of the EDCs in the final effluent;
- SA1 (tertiary treatment) was most effective in the removal of the xenoestrogens, SA2 and SA3 (both with aerobic bioreactors) had good removal efficiencies, while SA4 (anaerobic and aerobic lagoon system) had significantly lower removal efficiencies for NP and BPA;
- Removal of the steroidal estrogens was considerably lower at all four WWTPs, with SA2 having the most consistent in removal efficiency (*ca*. 60 % for E1, E2 and EE2);
- Estrogenic activity (measured by YES) was largely removed from WWTPs, irrespective of the treatment technology employed;
- Removal efficiencies of biological and physical performance indicators did not reflect the general removal efficiency of the EDCs;
- Concentrations of the EDCs in final effluents were reasonably constant among WWTPs, regardless of treatment technologies employed, possibly reflecting the combined effect of degree of loading, flow volume and treatment technology.

7.1.4 EDCs in WWTPs – influence of spatial and temporal sampling

In general, the concentrations of NP1EO, NP2EO and NP were high ngL-1 to low μgL^{-1}, while the remaining xenoestrogens and steroidal estrogens were in the low ngL^{-1} range in all surveyed WWTPs. The xenoestrogens, OP and BPA were usually detected at higher concentrations than the steroidal estrogens, while E1 was detected at higher concentrations than E2 and EE2. EE2 was usually detected at sub-ngL^{-1} levels, although its relative in vivo estrogenic potential makes these levels environmentally significant. Measured EEq values ranged from 0.05 to 2.78 ngL^{-1} and were always less than the EEq values predicted from the measured concentrations of the estrogens. This indicated some degree of anti-estrogenic activity was likely to be present in the collected WWTPs effluents. Based on the measured concentrations of the selected EDCs, the steroidal estrogens contributed overwhelmingly to the predicted EEq value.

Temporal variations in sampling also did not indicate any consistent differences in the concentrations of the selected EDCs. Therefore, despite the variation in climates, treatment plant technology, inputs into the wastewater stream or the populations serviced, the respective concentrations of the selected EDCs were generally consistent within and between WWTPs. While the measured EEq values were consistently lower than the predicted EEq values, the relationship of the measured and predicted EEq values were variable from among the WWTPs. There are several possible reasons for the observed disparity between predicted and measured EEq values. However, this mitigation in estrogenic response in the samples should be further assessed, including targeting potential compounds, such as weak estrogens and ER antagonists, which may have contributed to this effect.

Recent studies in Australia provide evidence that the concentrations represented in this current work indicate a reasonable reflection of baseline levels in the final effluent of Australian WWTPs. The consistency of the measured concentrations of the two 24 h composite samples could be largely coincidental, although it is worth investigating further whether the measured concentrations could represent common levels of EDCs that are released from Australian WWTPs. Conversely, the variability of measured EEq relative to predicted EEq values was also noted by Tan *et al*. (2007). This further indicates the potentially high variability of loading of so-called anti-estrogenic compounds unaccounted for by chemical analysis.

In summary:

- Based on 22 samples over two seasons from 11 WWTPs, the median concentrations measured for E1, E2 and EE2 were 23.9, 3.8 and 0.45 ngL^{-1}, respectively. Among the xenoestogens, BPA and OP were the compounds with lowest concentrations with median concentrations of 21.4 and 39.5 ngL^{-1} respectively. In contrast the median value of NP was much higher at 1114 ngL^{-1}. Similarly, the median concentrations for NP1EO and NP2EO were 1484 and 782 ngL^{-1}, respectively;
- Of the steroidal estrogens, concentrations of E1 were usually around an order of magnitude higher than E2 and EE2, while E2 was consistently higher than EE2;
- These concentrations were fairly consistent across WWTPs from different regions and the two sampling periods;

- The measured EEq values were consistently lower than the EEq values predicted from chemical concentrations. However, this relationship was found to be variable, indicating that the anti-estrogenicity in the effluents also varied considerably. Further work needs to be done to elucidate the reasons for this observed anti-estrogenicity.

7.1.5 EDCs in rural streams

Using ELISA, both E1 and E2 were detected in all samples taken from rural streams, including reference sites at high pgL^{-1} to low ngL^{-1} concentrations. Drains receiving dairy effluents had the highest concentrations of both E1 and E2, although concentrations in the receiving systems and even a reference sites were similar. Therefore, while the release of E1 and E2 from dairies exists, it is still unclear how significant this contribution is to the overall estrogenic potential of the receiving environment. As with the WWTP samples, E1 was detected at higher concentrations than E2, although the concentration ratio between E1 and E2 was consistently lower in the rural samples. Whether this can be used as an indication of the type of effluents that are entering a riverine systems warrants further investigation.

In general, higher levels of E1 and E2 led to higher EEq values, although there was a reasonable degree of variation between measured and predicted EEq values. However, both of the bioassays indicated the estrogenic activity measured in the samples was always less than that predicted by the measurement of E1 and E2 using ELISA. Based on further analysis of the samples using the YES assay, it is probable that the discrepancy was due to the presence of elements mitigating the estrogenic response in the samples. It would be difficult to determine what caused this apparent anti-estrogenic effect, although it is highly likely that different inputs would lead to the differences in variation between measured and potential estrogenic activity. For example, the Urban DC and rural Upstream EH samples both had a similarly lower measured EEq compared with the predicted EEq, although the cause of this discrepancy would be expected to be quite different. In the current investigation, a difference in the sensitivity was observed between the mammalian-based endogenous reporter gene assay (ER-CALUX) and the YES assay, demonstrating that the former can detect (xeno)estrogens at lower concentrations. The final choice of reporter gene assay to employ (mammalian-based or yeast-based) depends on the importance of lower detection limit versus ease of use and lower costs. A comparative assessment of various assays is desirable.

Overall, the results suggest that animal sources (e.g. dairy farming, piggeries, poultry and other concentrated animal operations) may contribute E1 and E2 to receiving aquatic systems, although further downstream the concentrations of E1 and E2 returned to levels that were detected at reference sites. This was also supported by the measured estrogenic activity, using both the YES and ER-CALUX bioassays. While the dataset of horticultural, stock grazing and urban areas were represented by fewer numbers of samples, it was apparent that the levels of E1, E2 and EEq were similar or slightly lower than the samples from dairy regions. While this work provides a preliminary indication of the potential contribution to estrogenic activity in rural waterways, a more comprehensive study would be required to gain further insight into the ecological risk posed by these activities. This would seem especially prudent regarding the environmental stressors, including drought and salinity that are already placing pressure on these riverine systems.

In summary:

- The steroidal estrogens, E1 and E2, were detected in all samples (including low but detectable levels at reference sites) and estrogenic activity was detected in all samples, except for three (all reference sites);
- The measured EEq was always less than the predicted EEq indicating elements mitigating estrogenic activity were present in the samples;
- The ER-CALUX bioassay was more sensitive in measuring estrogenic activity compared with the YES bioassay, although YES has a number of advantages for screening work, including cost and ease of use;
- Dairy farming, stock grazing, horticultural and urban activities (unrelated to sewage) can all contribute E1 and E2 to receiving aquatic systems.

7.2 General conclusions

- Sorption and degradation of EDCs are likely to play an important role in their fate within WWTPs and receiving systems. The extent to which these compounds can sorb to solids should be taken into consideration when sampling of aquatic ecosystems is undertaken;
- A range of steroidal estrogens and xenoestrogens can enter riverine systems through a variety of inputs, including effluents from WWTPs, dairy farms or diffuse sources such as in urban, horticultural and stock grazing areas;
- Treatment technologies used in WWTPs play an important role in reducing the total loading of xenoestrogens from influents. Removal efficiency of steroidal estrogens was highly variable between different treatment technologies (tested during winter in South Australia). However, concentrations in final effluents were consistent between WWTPs, despite the variations in the treatment technology used;
- The concentrations of selected EDCs and measured EEq values in WWTP effluents were similar to those reported in international surveys and recent Australian surveys. These findings suggest the concentrations detected are reasonable estimates of the levels of EDCs entering the aquatic environment from WWTPs;
- Based on the rural surveys, inputs of EDCs from human land use are likely to occur, particularly from concentrated animal operations, such as dairies, piggeries and other intensive animal husbandry activities;
- Anti-estrogenic effects are also likely to be present in effluent samples, potentially mitigating the estrogenic activity of estrogens that were both measured and not measured analytically. The discrepancy between the measured and predicted EEq values in the samples demonstrates the importance of using both biological and conventional analytical techniques.
- This exploratory research defines benchmarks of EDCs in some typical Australian environments, including those thought to be unaffected by effluent and those in the effluent stream of humans and dometic animals. EDCs are present in measurable amounts in Australian waste water treatment plant effluents at levels similar to those found in international surveys.
- As the understanding of EDCs impacts and management improves, through research projects such as this, it will become an increasingly tractable research, policy and regulatory issue.

8 Future research needs

This project was undertaken with a reasonably limited scope and, as expected, has identified a number of areas that require further knowledge. Furthermore, the areas that have been identified need to be prioritised in order to ensure the research undertaken addresses the most critical issues in a timely manner. Based on the data collated from this study, the following areas are worth further consideration to not only define the issue of EDCs in the Australian context but also to contribute to international research in this area.

- There are steroidal hormones being released in WWTP and feedlot effluents but whether these concentrations are sufficient to produce effects in fish living in rivers receiving effluent discharges is difficult to ascertain. However, in some rivers where the effluent contributes to a large volume of the flow, especially in dry summers it is possible that aquatic organisms may be exposed to concentrations of estrogenic chemicals sufficient to produce a biological response. This needs to be further investigated by conducting population level studies and by investigating wild fish that have been exposed to the effluent discharge. Recent Canadian studies involving whole-lake experiments (Kidd *et al*. 2007) are highlighting the need and urgency of studies assessing the impact of steroidal hormones in Australian aquatic ecosystems and, especially, on native fauna.
- In vitro assays, such as YES and ER-CALUX, are useful from a mechanistic standpoint and for use as screening tools for monitoring studies. However, the main disadvantage of these assays is their simplification of the *in vivo* situation. *In vivo* assays are vital as they permit the detection of effects that result from multiple mechanisms and also take into account processes such as bioavailability, toxicokinetics, metabolism and cross-talk between biological pathways. In Australia there is a need to develop *in vivo* test systems using our native fauna. As the aquatic environment is a common repository for many contaminants, the use of fish as a model test organism for evaluating the effects of contaminants is a logical choice. Currently molecular markers, including the distribution and localisation of the estrogen receptors (ERα and ERβ) and vitellogenin mRNAs in target tissues are being developed in a native fish species, the Murray rainbowfish, *Melanotaenia fluviatilis*, for potential use in the assessment of EDCs in the Australian riverine environment.
- Anti-estrogenic activity in treated effluents and their strong effect in masking the effect of known EDCs (e.g. E2) was a significant finding in this study, which was consistent with lower than expected (based on chemical assays) total loads of EDCs measured by bioassays. However, further investigation into anti-estrogenic activity is needed determine whether this represents a potential mitigating factor or whether it is related to the limitations of the bioassay. This should also be investigated using common sample preparation between assays.

- The selected EDCs and the anti-estrogenic effect highlight the main limitation of this work, in that an a priori selection of analytes will only reveal a fraction of the contaminants that are part of an effluent stream. Not only do the effects of contaminant mixtures (multistressor) on ecosystem health need to be better understood but also the role of contaminants in suppressing or enhancing exposure and the toxicological impact of EDCs should be established.
- Despite short half-lives observed in this study, some of the EDCs may accumulate in aquatic systems (especially in anoxic sediments or groundwater aquifers) receiving a continuous discharge of effluents. Levels of EDCs in sediments were not tested in the current project and currently little data is available for sediments in Australia. However, based on observations from overseas studies, sediments in aquatic ecosystems are expected to accumulate a range of compounds and the consequences of exposure to benthic organisms of EDCs needs to be established. Among such compounds, polybrominated flame retardants (PBDEs) are highly relevant due to their increasing concerns to wildlife and human health and their ability to bioaccumulate and survive in sediment.
- The environmental fate of estrogenic compounds in sediments and soils has so far been studied under controlled and optimum conditions for biological activity. The environmental fate of these compounds in real field situations, in situ and in the presence of other co-contaminants need to be investigated under a range of climatic conditions. Furthermore, the role of antimicrobial agents and other co-contaminants on the environmental fate of EDCs in sediments is urgently needed.
- A range of bioassays (E screen, ER-CALUX, YES) are available and various laboratories are using one or the other. A comparative study should be performed to seek the recommended set of bioassay covering different mode of actions, to isolate confounding factors, to interpret the results and clarify their role as bioanalytical tools in monitoring studies. Furthermore, little work has been undertaken assessing the extent of androgenic activity in aquatic systems using an assay such as YAS. As with the present study, such an assessment should be undertaken in conjunction with chemical analytical techniques.
- Although only a limited number of samples were tested in the current study, the data indicate that animal operations (dairy, piggeries, feedlots, and poultry farms) may be a significant source of EDCs in riverine environment. However, such effluents are generally dispersed on land through irrigation and not discharged directly into the riverine environment. Therefore, the environmental fate of EDCs in soils receiving effluents and manure wastes and their potential off-site migration (e.g. runoff) and impact on ecosystems needs to established.
- Low cost treatment options should be explored that are particularly suitable for rural WWTPs. The finding from the biodegradation study indicating that key EDCs present in reclaimed wastewater or treated effluent are readily degradable in aerobic biologically active environments should be utilised as a basis of a land-based treatment system.
- Successful transfer of EDC research methods and findings from international studies and researchers has enabled EDC understanding to grow efficiently in Australia and underscores the value of international perspectives and exposure to natural resource management issues.
- There remains, beyond the scope of this research project but closely linked to the field, an important awareness and information gap that needs to deal with the way society and individuals deal with EDCs.

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Appendix 1

Quality assurance and quality control in field sampling

Site selection

Site reconnaissance visits were made and survey of potential sampling spots was carried out with help from collaborators, such as the Queensland EPA and South Australian EPA. The representative nature of sampling sites, potential confounding factors, occupational health and safety and other potential issues were taken into account when sampling sites were selected. In general, the protocols suggested by United States Geological Survey (USGS, 1999) in the National Field Manual for the Collection of Water-Quality Data were used. This included protocol being developed for personal hygiene to avoid the use of personal care products (i.e. insect repellents such as DEET, colognes, perfumes), caffeinated products and tobacco by the sampling team during sample collection and processing.

Preparation of sampling containers

Bottles used for sampling were thoroughly prepared prior to collection of samples. An overview of this procedure is as follows:

1. Soak each bottle and cap in a Pyroneg detergent solution for16 hours
2. Decant half of the detergent solution in each bottle.
3. Shake vigorously for a minute and decant.

4 Rinse off any soap residue in the bottle using tap water.

5. Fill each bottle with _ full of deionised water.
6. Shake vigorously and decant.
7. Repeat deionised water rinse › 3 times.
8. Fill each bottle with _ full of Milli-Q water.
9. Shake vigorously and decant.
10. Repeat Milli-Q water rinse 3 times.
11. Rinse each bottle (1 L) and cap with 25 mL (each time) of HPLC grade acetone 3 times; inverting bottle to ensure mixing.
12. Rinse each bottle and cap with 25 mL (each time) of HPLC grade methanol 3 times.
13. Bake glassware at 350°C
14. Store bottles firmly capped

Colour-coded labels were used to differentiate acidified samples for the chemical analyses from the samples for the biological analyses, which did not need any prior manipulation. The labels contained information on the date of collection; time of collection, site name, sample type (WWTP, outfall, up-stream/down-stream) and location determined by GPS, where possible.

Collection of samples

Replicate 1 L samples were collected at each sites for each analytical procedure (i.e. GC-MS /ELISA and YES / ER-CALUX). Duplicate blind and blank samples were also collected by the respective collaborating agency in each state. For the blank samples, two bottles filled with Milli-Q water were taken to the field to check for potential contamination. Table 1a shows blank samples collected from Queensland WWTPs for the two sampling periods. NP was consistently the EDC detected at the highest concentrations in the blank samples. Where the concentrations were significantly higher than the LOQ, then the collected samples were not used. This was done for one batch of samples from ACT and a number of samples from SA.

Blind samples were randomly selected by the collaborating agency collecting the samples, along with the samples i.e. an additional replicate sample was included during the sampling procedure. The location of the blind samples was not revealed to the analytical laboratory prior to analysis. An example of blind samples taken from the ACT WWTPs demonstrating the comparisons between blind samples and labelled samples are given in Table 1b. These samples given as examples were taken from other areas of the WWTP and are not comparable with the effluent samples.

Table 1a
Concentration in ngL^{-1} of xenoestrogens in field blanks collected from Queensland WWTPs, following GC-MS analysis.

Compound	Sample								
	Q1 a	Q5 a	Q2 a	Q3 a	Q4 a	Q4 b	Q5 b	Q5 c	Q1 b
OP	29	32	29	31	17	21	21	34	37
NP	128	205	162	213	250	36	51	42	47
NP1EO	30	‹LOQ	34	‹LOQ	‹LOQ	‹LOQ	‹LOQ	‹LOQ	‹LOQ
NP2EO	70	54	39	‹LOQ	‹LOQ	36	30	30	38
BPA	‹LOQ	17	17	27	17	17	17	18	18

Table 1b
Concentration in ngL^{-1} of xenoestrogens comparing two blind samples with labelled samples collected from ACT WWTPs, following GC-MS analysis.

Compound	Sample a		Blind sample a		Sample b		Blind sample b	
	Mean	S.D. [a]	Mean	S.D.	Mean	S.D.	Mean	S.D.
OP	204.6	1.34	216.7	18.48	56.5	4.5	54	1.4
NP	2928.7	33.06	2713.1	266.59	497	400.5	544.5	111.0
NP1EO	4146.9	337.91	4007.6	465.89	675.5	636.5	723	22.6
NP2EO	5802.7	265.95	5480.7	662.33	317	172.5	249.5	75.7
BPA	428.1	35.18	407.6	17.79	0.5	0	0	0

a Standard deviation

Sample extraction

Prior to filtration and extraction, the surrogate 4-*n*-NP was spiked to the samples to monitor the consistency of the sample preparation, up to analysis. Recovery tests with the SPE step were assessed with 100 ngL^{-1} solutions of the EDCs, spiked to deionised water, passed through the SPE cartridges. The recoveries were good using deionised water (Table1c), although recoveries became more variable when the 4-*n*-NP was spiked to effluent samples and ranged from 60 to 123 %.

Table 1c
Percent recovery of selected EDCs from deionised water.

SPE Brand	Compound							
	BPA	OP	NP	NP1EO	NP2EO	E1	E2	EE2
Waters C18	108.4±2.6	104.9±1.1	11.7±1.5	81.6±3.5	79.6±3.3	108.8±1.8	98.7±2.5	106.5±1.8
Oasis HLB	97.4±2.6	93.0±2.4	101.6±1.4	89.1±0.8	87.3±2.3	95.9±3.9	94.1±0.3	100.3±1.5

Appendix 2

Analysis by GC-MS

GC-MS was used in this project for the analysis of steroidal estrogens and xenoestrogens in sorption and degradation experiments. GC-MS was also used for the xenoestrogens only for analysis of samples collected from WWTPs.

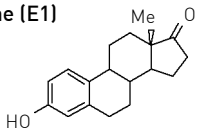
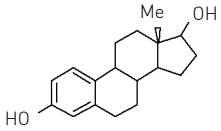
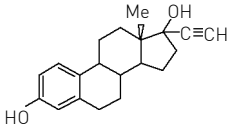
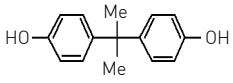
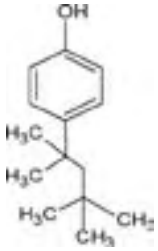
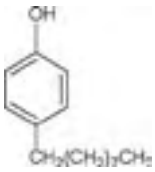
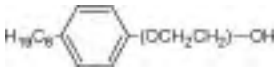
In all GC-MS determinations, analytes were converted into their trimethylsilyl analogues using N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) by reacting at 80°C for 60 min. Silylation is a popular derivatisation method for organic compounds containing active hydrogen atoms (e.g. –OH), which converts the organic compound to a derivatised product with reduced polarity, enhanced volatility and increased thermal and catalytic stability.

A typical GC-MS total ion chromatogram (TIC) of trimethylsilyl (TMS) derivatives obtained by running a mixed standard containing all of the analytes used in this study is given in Figure 2a. The corresponding mass spectra for each of the analytes are given in Figure 2b, which shows typical fragmentation patterns for the TMS derivatives. Table 2 presents a summary of the retention times and the typical fragment ions. The order of retention and the fragmentation patterns observed were representative of those found in the literature (Shareef *et al.* 2004; Ying and Kookana 2005; Shareef *et al*.2006).

Single quadrupole MS can have a number of limitations associated with analysing environmental samples, which means quantification of an analyte requires a number of criteria to be met (Ingerslev and Halling-Sorensen 2003). For quantification of samples, ions identified as quantification ions were used along with at least two qualifying (or confirmatory) ions, including the molecular ion of the derivatised compound. The criteria used for identification of compounds within samples were:

1. retention times of the unknown peaks matched that of peaks obtained from standards;
2. the mass spectra obtained contained the molecular ion of the derivatised compound;
3. the mass spectra for the unknown peaks in samples matched those obtained for standards with the correct relative intensities of the two or more ions selected for qualification.

Table 2
GC-MS parameters for identification and quantification of selected EDCs in samples

Analyte	Molecular Weight	Retention Time (min)	Quantitation Ion	Qualifying Ions
Estrone (E1) 	270	18.6	342*	73,218,244,257
17β-estradiol (E2) 	272	18.9	285	73,231,327,416*
17α- ethynylestradiol (EE2) 	296	19.9	425	285,300,440*
Bisphenol A (BPA) 	228	15.9	357	73,191,217,372*
4-*t*-octylphenol (OP) 	206	11.8	278*	207,263
4-nonylphenol (NP) 	220	14.0	179	73, 277, 292*
NP-ethoxylate (NP1EO) 	264	14.8-15.4	265	251,336, 307*
NP-ethoxylate (NP2EO) 	308	16.4-17.0	295	281, 309,351*

***** Molecular ion of derivatised compound

Figure 2a
GC-MS TIC of trimethylsilyl (TMS) derivatives of the estrogens and xenoestrogens

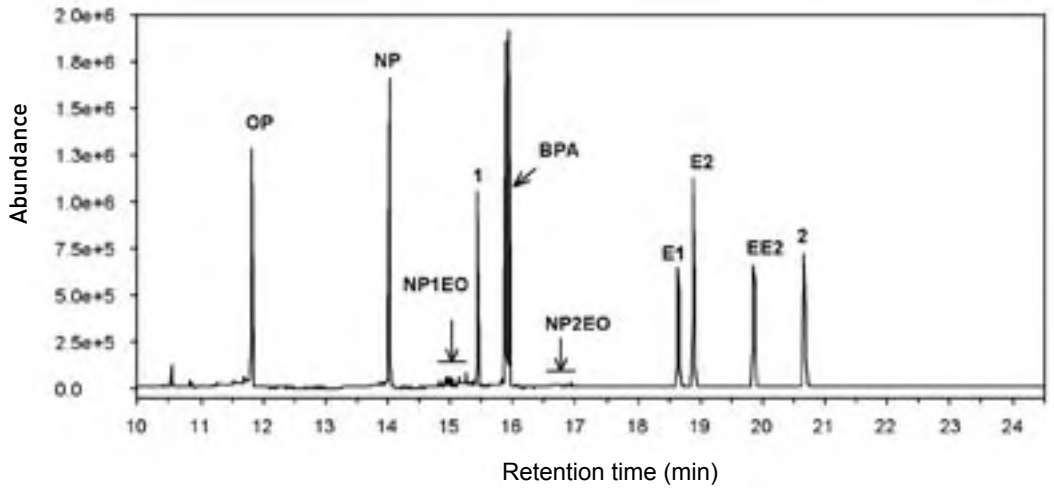
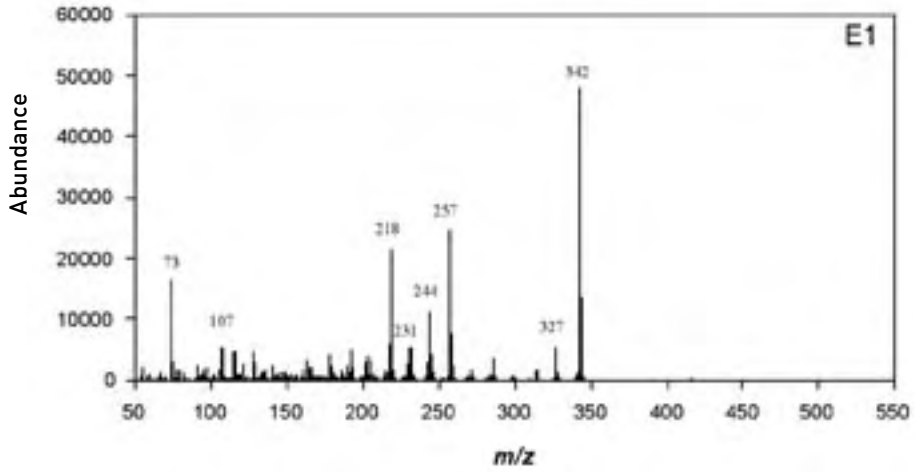
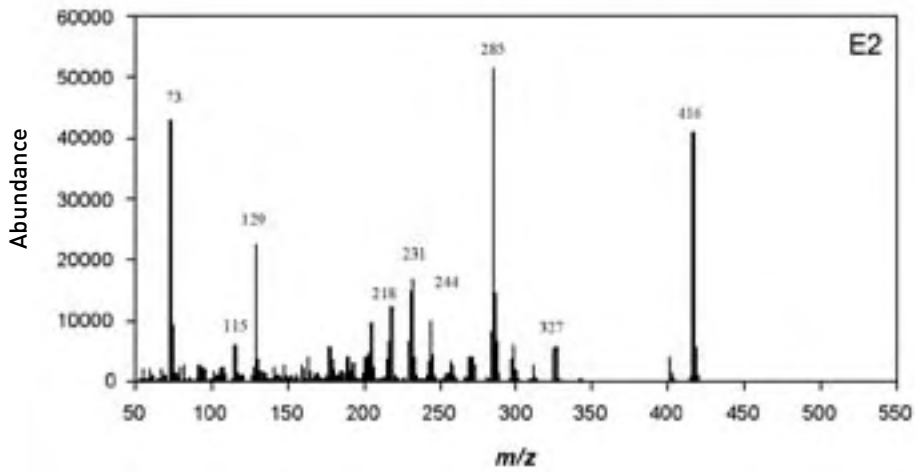
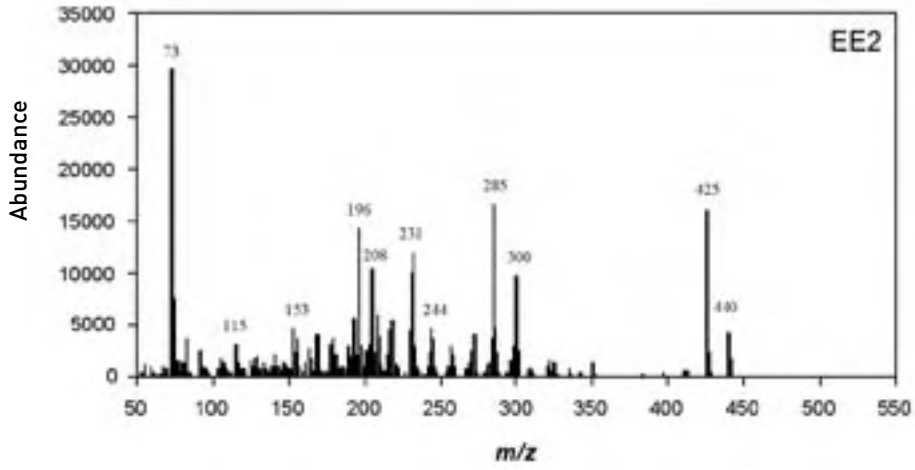
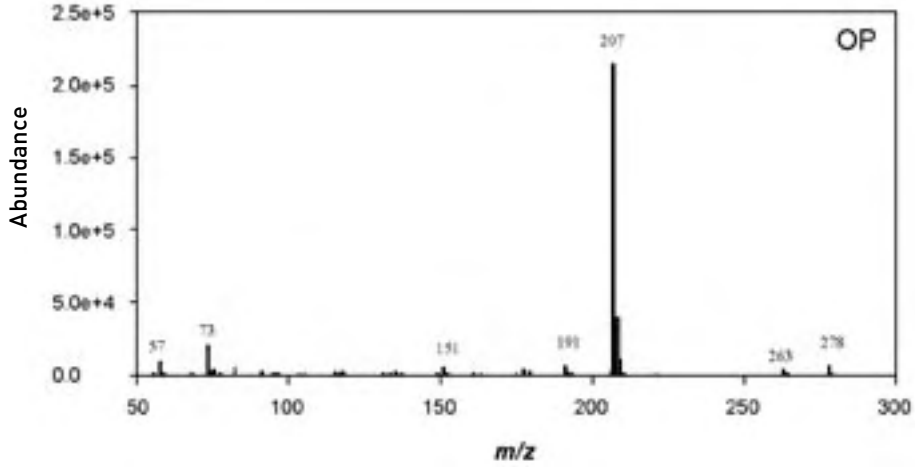


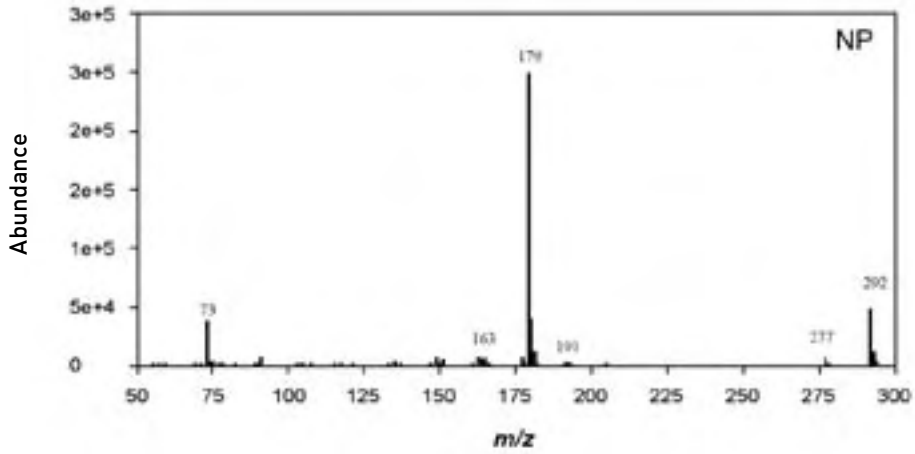
Figure 2b
Mass spectra of trimethylsilyl (TMS) derivatives of the estrogens and xenoestrogens selected for this study. The proposed fragmentation pattern of TMS-E1 is shown with its mass spectrum as an example of typical fragmentation.






EE2
Abundance
m/z
35000
30000
25000
20000
15000
10000
5000
0
50
100
150
200
250
300
350
400
450
500
550
73
115
153
196
208
231
244
285
300
425
440


OP
Abundance
m/z
2.5e+5
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0.0
50
100
150
200
250
300
57
73
151
191
207
263
278


NP
Abundance
m/z
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3e+5
2e+5
2e+5
1e+5
5e+4
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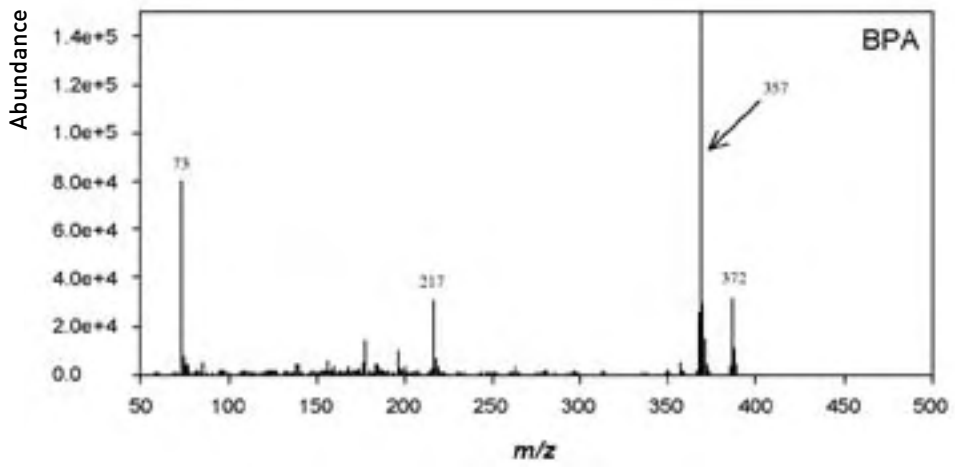
75
100
125
150
175
200
225
250
275
300
73
163
191
170
277
292


BPA
Abundance
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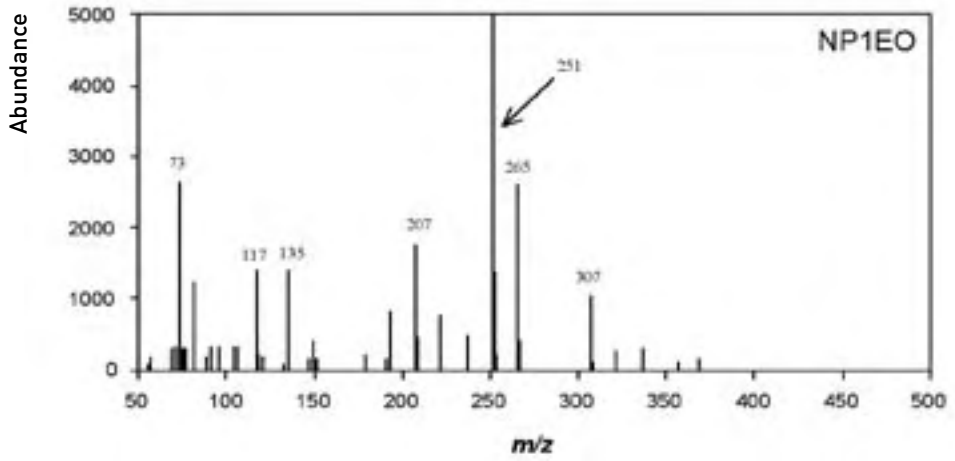
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
73
217
357
372
50
100
150
200
250
300
350
400
450
500
m/z


NP1EO
Abundance
5000
4000
3000
2000
1000
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73
117
135
207
251
265
307
50
100
150
200
250
300
350
400
450
500
m/z

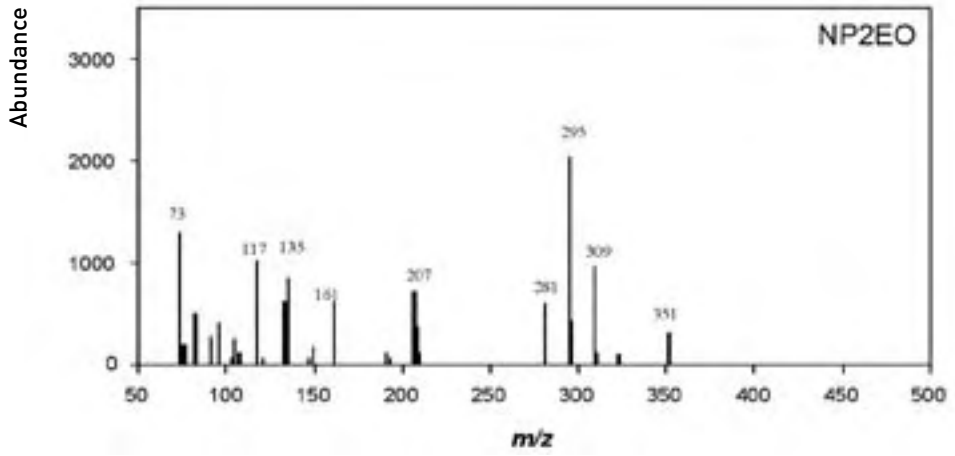
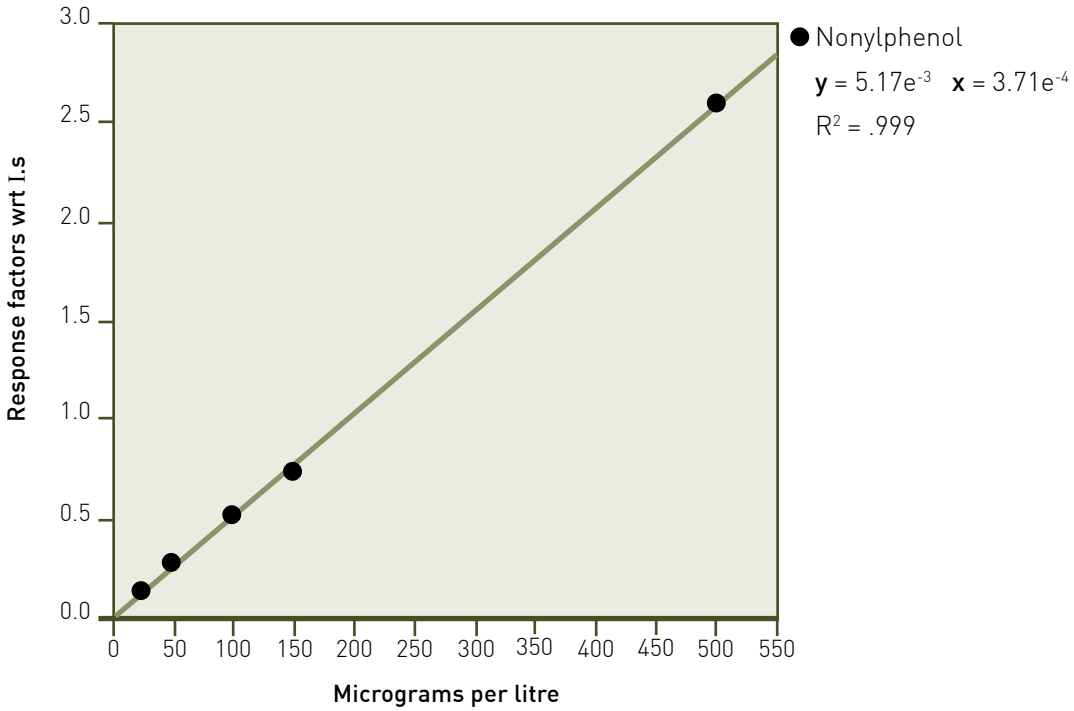

NP2EO
Abundance
3000
2000
1000
0
73
117
135
161
207
281
295
309
351
50
100
150
200
250
300
350
400
450
500
m/z

Figure 2C
A typical GCMS calibration curve obtained for the trimethylsilyl derivative of 4-nonylphenol (TMS-NP).



Appendix 3

ELISA

ELISA procedure

The ELISAs were carried out according to the manufacturer's instructions. Briefly, each sample was prepared in 10% (v/v) methanol / water and run in duplicate. The sample was mixed with enzyme conjugate (1:1 ratio) and 100 mL of this mixture was transferred to the ELISA plate and incubated for 1 h at room temperature. The plate was washed three times with washing buffer followed by the addition of the substrate. After 30 minute incubation, the reaction was stopped and the plates were read at an absorbance of 450 nM. Each plate consisted of duplicate standards (E1 at 0.05 to 5 μgL^{-1}, E2 at 0.05 to 1 μgL^{-1}or EE2 at 0.05 to 3 μgL^{-1}) and blanks. Where needed, dilutions of the original samples were conducted to ensure the response was within the analytical range of the standard curve. Only sample responses that fell within the analytical range were included in the analysis. Concentrations in the samples were based on the responses obtained from standard curve analysis. According to the manufacturer, the detection limit for E1, E2 and EE2 was 0.05 μgL^{-1}. However, with sample pre-concentration a detection limit of 0.05 ngL^{-1} for each compound was achieved.

Calculation of E1, E2 and EE2 from the ELISA using the Hill Equation

Nonlinear regression using a four parametric logistic curve fit was applied to each of the standard responses. The Hill equation was used to estimate the concentration of E1, E2 or EE2 in samples based on the response of the standards (SigmaPlot., version 10, SPSS).

The Hill equation is defined as:

Concentration = C*(((A-D)/('OD'-D)-1)(1/B))

where, A=maximum, B=slope, C=EC50, D=minimum and OD=optical density.

Table 3a
E1 example calculation

Sample ID	OD [a]	Hill Equation [E1] (μgL^{-1})	Concentration Factor [b]	[Sample] (ngL^{-1})
A	0.255	3.350	100	33.5
B	0.235	3.650	100	36.5

a Optical density
b 1000 mL sample concentrated to 1 mL extract, 1/10 dilution of extract in Milli Q used in ELISA

Figure 3a
Standard curve for E1 standard. Each data point is the average response ± SD (n=2). Four parametric logistic curve fit, slope=0.7195, r^2=0.9961.

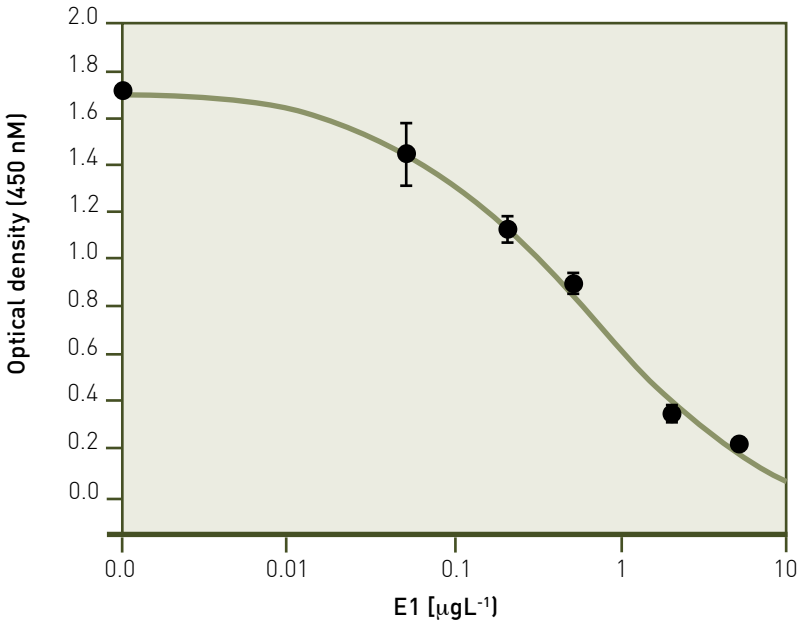


Table 3b
E2 example calculation

Sample ID	OD [a]	Hill Equation [E2] (μgL^{-1})	Concentration Factor [b]	[Sample] (ngL^{-1})
A	0.492	0.330	100	3.3
B	0.465	0.350	100	3.5

a Optical density
b 1000 mL sample concentrated to 1 mL extract, 1/10 dilution of extract in Milli Q used in ELISA

Figure 3b
Standard curve for E2 standard. Each data point is the average response ± SD (n=2). Four parametric logistic curve fit, slope=1.0083, r^2=0.9784.

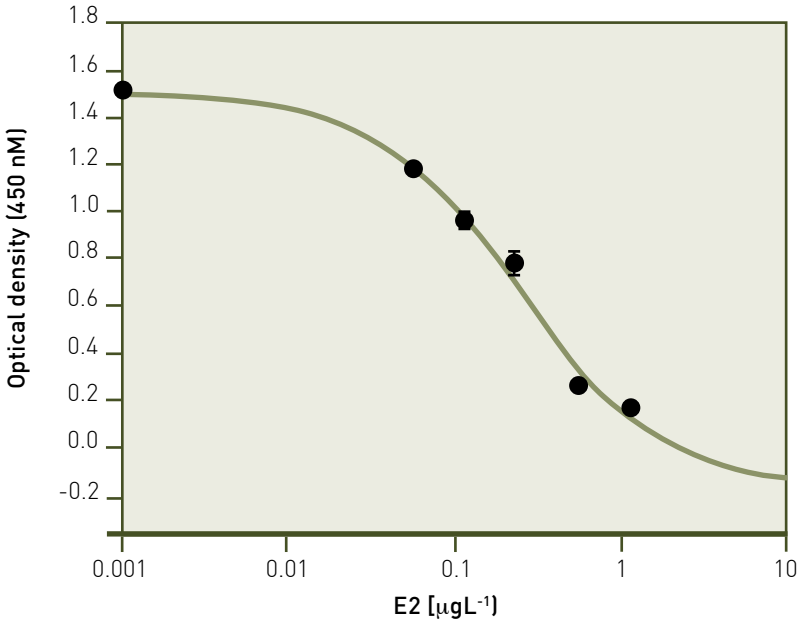
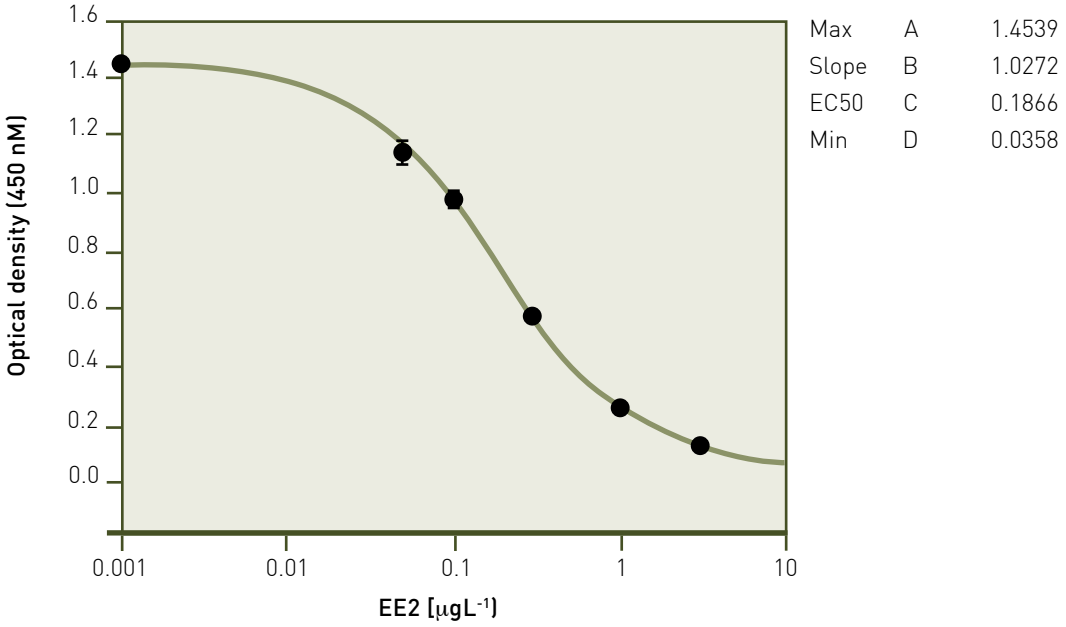


Table 3c
EE2 example calculation

Sample ID	OD [a]	Hill Equation [EE2] (μgL^{-1})	Concentration Factor [b]	[Sample] (ngL^{-1})
A	1.120	0.059	100	0.59
B	1.030	0.081	100	0.81

a Optical density
b 1000 mL sample concentrated to 1 mL extract, 1/10 dilution of extract in Milli Q used in ELISA

Figure 3c
Standard curve for EE2 standard. Each data point is the average response ± SD (n=2). Four parametric logistic curve fit, slope=0.1.0272, r^2=0.9983.



Appendix 4

YES assay

YES background and procedure

For this assay, the human estrogen receptor a (hERα) is stably transfected into the yeast genome, *Saccharomyces cerevisiae*, along with an expression plasmid carrying estrogen response elements (EREs) and the reporter gene, *Lac-Z* (encoding the enzyme β-galactosidase). Upon binding an active ligand, the E2-occupied receptor interacts with EREs to modulate the expression of *Lac-Z*. β-galactosidase is synthesised and secreted into the medium where it metabolises the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG) from yellow to a red product. This colourimetric change is measured at an absorbance of 540 nm. The relative binding of estrogenic compounds are compared with the standard binding of 17β-estradiol (E2).

Following filtration and SPE, samples were serially diluted by a factor of 2, with 6 diluted samples produced, in absolute ethanol in sterile 96-well microtitre plates. From each dilution 50 mL was transferred to another plate and left to evaporate to dryness. In addition to detecting estrogenic compounds, YES can also be used to detect antagonism of the hER (anti-estrogenicity) in samples. The anti-estrogenic response was undertaken following the method used by Sohoni and Sumpter (1998). Briefly, E2 was set at a sub-maximal response (where half the maximum colour saturation occurred) and the ability of a chemical to inhibit this response was determined. This assessment of antagonism was undertaken for all collected samples.

Each plate contained one standard row (positive controls E2 at 1.3 ngL^{-1} to 2.7 μgL^{-1} for YES), one row of blanks (ethanol and assay medium only) and one row of E2 at the sub-maximal response. All samples were run in duplicate. Seeded assay medium (200 μL aliquots) was added to each sample well. Plates were sealed and incubated for three days at 32°C. The plates were read at 540 nm (for CPRG) and at 620 nm (for yeast turbidity). If samples exhibited gross toxicity to the yeast, based on the measurement of yeast cell density, samples were diluted further until no toxicity to the yeast was observed.

YES calculations: corrected absorbance

The YES response (corrected absorbance) was calculated by subtracting the absorbance of the yeast (read at 620 nm) from the absorbance of the substrate (read at 540 nm) for both test wells and blank wells. Furthermore, the response of the blank wells was subtracted from the test wells. The response was expressed as a corrected optical density (OD) value, eliminating the background expression of β-galactosidase (β-gal) naturally produced by yeast cells. The blank wells contain all the components of the test wells except the test chemical, therefore difference in response of the test wells can be attributed to the presence of the test chemical and not the diluent used (ethanol).

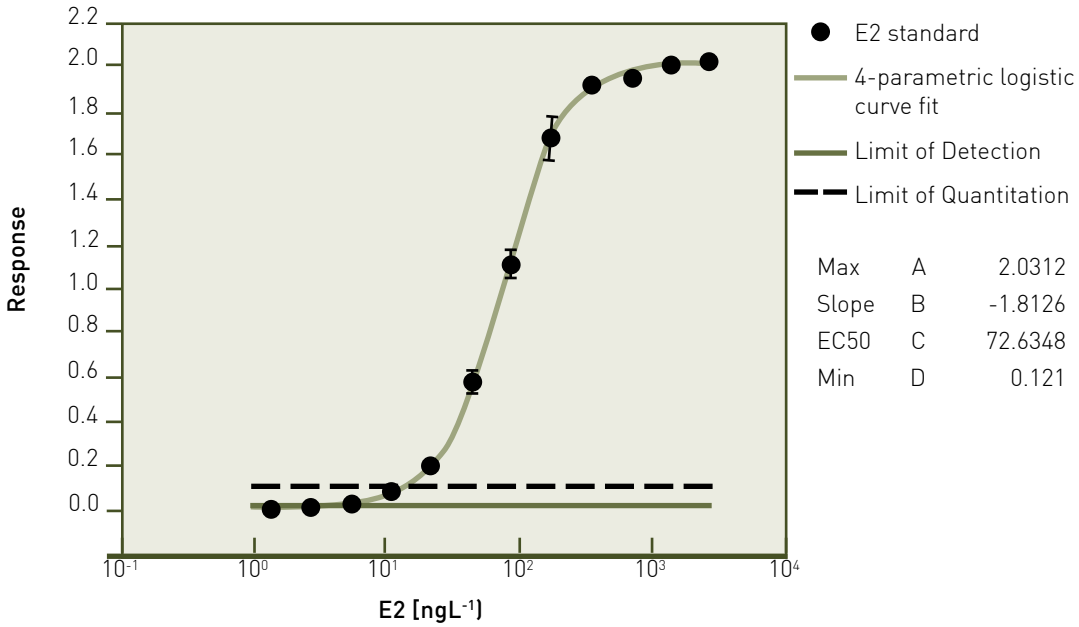
$$R = Sample\,[OD_{540} - OD_{620}] - blank[OD_{540} - OD_{620}]$$

is used to calculate the response (R) for the optical density of the sample and blank at 540 nm (OD_{540}) and 620 nm (OD_{620}).

Limit of detection (LOD)/limit of quantification (LOQ) of the YES

A signal-to-noise ratio of three (3x the standard deviation of the response of the blank samples) was used for the LOD and a ratio of ten (10x the standard deviation of the response of the blank samples) was used for the LOQ. The LOD and LOQ were calculated for each E2 standard curve and the figure below is a representative E2 standard dose-response curve with the LOD and LOQ. In this case, the assay LOD was 6.5 ngL^{-1} and 0.13 ngL^{-1} with pre-concentration, while the assay LOQ was 15.1 ngL^{-1} and 0.3 ngL^{-1} with pre-concentration (values are sample concentration factor dependent). The lowest LOQ, following sample pre-concentration, was 0.05 ngL^{-1}.

Figure 4a
Representative dose-response curve for E2 standard. Each data point is the average response ± SD (n=3). Four parametric logistic curve fit. The LOD defined as 3x signal-to-noise ratio and LOQ defined as 10x signal-to-noise ratio.



Calculation of estradiol equivalents (EEqs) in the YES

The response from the sample with an unknown content was compared to the response of E2. Six dilutions of the effluent extract (2-fold dilution factor) were made and tested in the YES. Where 100% extracts exhibited gross toxicity to the yeast a 10-50 x dilution was made prior to the six dilutions included in the YES. To calculate the EEqs the lowest two to three effluent dilutions were used due to the non-parallel nature effluent dilutions and E2 in the YES.

Nonlinear regression using a four parametric logistic curve fit was applied to the E2 standard curve. The Hill equation was used to estimate the EEq in samples based on the response of the standard (SigmaPlot, version 10, SPSS).

The Hill equation is defined as:

Concentration = C*(((A-D)/('OD'-D)-1)(1/B))

where, A=maximum, B=slope, C=EC50, D=minimum and OD=optical density.

Table 4a
EEq sample calculation

Sample ID	Percent extract	Response	Hill Equation [Well EEq] (ngL^{-1})	Concentration Factor [a]	[Sample EEq] (ngL^{-1})
A	2.5%	0.328	26.8	25	1.1
B	1.25%	0.202	18.8	12.5	1.5

a 1000 mL sample concentrated to 1 mL extract, 1/10 dilution of extract in methanol, 50 µL used in YES

Appendix 5

Predicted EEqs from chemical analysis

The predicted EEq of the effluents were calculated as the sum of individual chemical concentrations (C_i), determined from ELISA, and multiplied by their respective estrogenic equivalency factor (EEF):

$$EEq = \sum\{C_i \, xEEF\}$$

The relative potencies (to E2) of each compound used to calculate the predicted EEqs were based on the YES and are listed in the following table:

Table 5a
Relative *in vitro* potency of selected estrogens based on the yeast estrogen screen (YES)

Compound	Relative Potency to E2 (*in vitro*)
17β-estradiol (E2)	1.0 [a]
Estrone (E1)	0.22 [a]
17α-ethynylestradiol (EE2)	1.03 [a]
Bisphenol A (BPA)	0.0001 [a]
4-*tert*-octylphenol (OP)	0.0004 [a]
4-*n*-nonylphenol (4-*n*-NP)	0.000009 [a]
4-*tert*-nonylphenol (4-NP)	0.00014 [a]
Nonylphenol ethoxylates (NP1EO, NP2EO)	0.000006 [b]

a based on yeast estrogen screen (Woods, 2007), **b** (Jobling and Sumpter, 1993)

Appendix 6

Summary of temporal and spatial trends of concentrations of selected EDCs and EEq in final effluents of WWTPs

	Compound																			
WWTP	E1		E2		EE2		BPA		OP		NP		NP1EO		NP2EO		EEq (Predicted)		EEq (Measured)	
	S1 [a]	S2 [b]	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
ACT Region																				
ACT 1	20.6± 2.4	22.3	1.23± 0.01	5	0.24± 0.01	0.78	27± 3	20	49± 6	64± 3	1536± 66	893± 27	6616± 673	2891± 199	2079± 23	1395± 122	6.06± 0.54	10.72± 0.49	1.40± 0.38	1.96± 0.88
ACT2	5.76± 2.29	3.14	‹ LOQ	1.42	‹ LOQ	0.15	7± 8	16± 1	60± 5	18	320± 42	514± 2	768± 86	160± 29	264± 241	158± 26	1.35± 0.51	2.28± 0.01	0.75± 0.09	0.15± 0.03
QLD Region																				
Q1	9.12± 1.58	29.1± 0.54	1.37± 0.11	5.69± 0.51	0.14± 0.01	1.14± 0.32	18± 2	13	26± 4.8	49± 8	1054± 32	614± 57	937± 185	1556± 83	412± 58	978± 186	3.54± 0.47	13.47± 0.96	0.03± 0.01	1.91± 1.00
Q2	9.30± 1.17	21.3± 2.06	1.57± 0.03	3.73± 0.11	0.11± 0.02	0.57± 0.02	15± 1	31± 6	30± 4	62	1404± 89	1029± 172	1407± 157	2224± 28	304± 115	684± 57	3.76± 0.31	9.05± 0.59	0.28± 0.13	0.54± 0.13
Q3	25.5± 0.84	25.7± 0.41	1.84± 0.06	6.35± 0.14	0.36± 0.09	1.20± 0.04	25	20± 1	46± 6	165± 6	1889± 143	1679± 167	2219± 188	2650± 353	293± 19	1181± 75	7.88± 0.54	13.34± 0.28	0.18± 0.07	0.21± 0.05
Q4	25.9± 9.17	17.6± 0.58	1.64± 0.26	3.60± 0.35	0.25± 0.04	0.75± 0.03	20± 4	20± 9	45± 6	165± 6	1333± 207	947± 20	1764± 150	1124± 33	2987± 224	4432± 109	7.64± 2.32	8.29± 0.51	0.66± 0.05	0.05± 0.02
Q5	32.2± 0.59	32.1± 3.89	1.39± 0.07	4.71± 0.09	0.40	0.71± 0.01	44± 6	26± 4	56± 5	17± 3	2991± 349	1041± 50	795± 164	1477± 106	379± 47	735± 196	8.94± 0.21	12.54± 0.96	0.05± 0.03	0.12± 0.04
South Australian Region																				
SA1	39.3± 0.94	30.6± 2.54	4.20± 0.19	6.22± 0.63	0.40± 0.11	‹ LOQ	50± 4	4± 1	12± 2	11± 1	1525± 182	1412± 141	3980± 98	949± 258	2373± 204	1449± 303	13.28± 0.51	13.03± 1.20	0.15± 0.07	1.31± 0.22
SA2	18.2± 0.37	20.9± 0.76	3.60± 0.33	6.18± 0.87	0.50± 0.01	0.28± 0.07	15	‹ LOQ	13± 1	35	860± 78	599± 144	2185± 107	1536± 93	476± 25	773± 95	8.13± 0.42	11.10± 1.11	0.05± 0.02	1.09± 0.65
SA3	37.6± 2.52	12.7± 2.62	2.80± 0.13	5.98± 0.55	1.30± 0.08	0.19± 0.04	23± 1	‹ LOQ	41± 3	28± 1	2887± 314	635± 27	1451± 72	839± 405	1221± 162	424± 148	12.46± 0.99	8.99± 1.17	0.96± 0.26	1.46± 0.37
SA4	34.2± 0.31	30.9± 0.8	3.90± 0.06	3.83± 0.55	0.60± 0.04	0.41± 0.07	148± 1	127± 2	37± 6	13± 1	1415± 63	1564± 44	1256± 57	390± 30	1099± 170	484± 10	12.09± 0.17	11.09± 0.81	1.85± 0.88	2.21± 0.75

a Sampling period 1, **b** Sampling period 2