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An assessment of endocrine activity in Australian rivers using chemical and in vitro analyses

Philip D. Scott[1], Michael Bartkow[2], Stephen J. Blockwell[3], Heather M. Coleman[4], Stuart J. Khan[4], Richard Lim[5], James A. McDonald[4], Helen Nice[6], Dayanthi Nugegoda[7], Vincent Pettigrove[8], Louis A. Tremblay[9,10], Michael St. J. Warne[11], and Frederic D.L. Leusch[1,*]

[1] School of Environment, Griffith University, Southport, Queensland, 4222, Australia

[2] Seqwater, PO Box 16146, Brisbane City East, Queensland, 4002, Australia

[3] Sydney Water Corporation, PO Box 399, Parramatta, New South Wales, 2124, Australia

[4] School of Civil & Environmental Engineering, University of New South Wales, New South Wales, 2052, Australia

[5] School of the Environment, University of Technology Sydney, PO Box 123, Broadway, New South Wales, 2007, Australia

[6] Water Science Branch, Department of Water, Government of Western Australia, PO Box K822, Perth, Western Australia, 6842, Australia

[7] School of Applied Sciences, Royal Melbourne Institute of Technology, PO Box 71, Bundoora, Victoria, 3083, Australia

[8] Melbourne Water, PO Box 4342, Melbourne, Victoria, 3001, Australia

[9] Cawthron Institute, 98 Halifax Street East, Nelson, 7042, New Zealand

[10] School of Biological Sciences, University of Auckland, PO Box 92019, Auckland, 1142, New Zealand

[11] Water Quality and Investigations, Department of Science, Information, Technology, Innovation and the Arts, Queensland Government, GPO Box 5078, Brisbane, Queensland, 4001, Australia

* Corresponding Author.

e: f.leusch@griffith.edu.au

p: +61 7 5552 7832
Mailing address:
Dr Frederic Leusch
Smart Water Research Centre (G51)
Griffith University, Gold Coast Campus
Southport, Queensland, 4222
Australia

Abstract

Studies on endocrine disruption in Australia have mainly focused on wastewater effluents. Limited knowledge exists regarding the relative contribution of different potential sources of endocrine active compounds (EACs) to the aquatic environment (*e.g.*, pesticide run-off, animal farming operations, urban stormwater, industrial inputs). In this study, 73 river sites across mainland Australia were sampled quarterly for one year. Concentrations of 14 known EACs including natural and synthetic hormones and industrial compounds were quantified by chemical analysis. EACs were present in 88% of samples (250 of 285). Bisphenol A was the most frequently detected EAC (66%) and it's predicted no-effect concentration (PNEC) was exceeded 24 times. The most common hormone was estrone, present in 28% of samples, and the PNEC was also exceeded 24 times. Ethinylestradiol was detected in 10% of samples at concentrations ranging from 0.05 - 0.17 ng/L. It was detected in many samples with no wastewater influence, and the PNEC was exceeded 13 times. In parallel to the chemical analysis, endocrine activity was assessed using a battery of CALUX bioassays. Estrogenic activity was detected in 19% (53 of 285) of samples (LOQ=0.1 ng/L 17β-estradiol equivalent; EEQ). Seven samples had estrogenic activity (1 - 6.5 ng/L EEQ) greater than the PNEC for 17β-estradiol. Anti-progestagenic activity was detected in 16% of samples (LOQ=8 ng/L mifepristone equivalents; MifEQ), although the causative compounds remain unknown. With several compounds and endocrine activity exceeding PNEC values, there is potential hazard to the Australian freshwater environment.

Keywords

AR-CALUX; EDC; endocrine disruption; ER-CALUX; ethinylestradiol; PR-CALUX

1. Introduction

Since the early 1980s, there has been increasing evidence that endocrine active compounds (EACs) negatively affect fish reproduction and pose a risk to ecological and human health (McLachlan et al. 1984; WHO/IPCS 2002). The first evidence of endocrine disruption in the aquatic environment was reported in the 1980s, when masculinized female mosquitofish (*Gambusia holbrooki*) were identified up to 6.5 km downstream of a pulp mill effluent discharge (Howell et al. 1980). Additional research has since demonstrated that endocrine disruption in aquatic environments is a widespread problem (Campbell et al. 2006; Jobling et al. 1998; Sumpter 2005; WHO/IPCS 2002) and often associated with chemicals present in municipal and industrial wastewater discharges.

The first evidence of endocrine disruption in Australian rivers was reported in 1999 when male mosquitofish captured downstream of a wastewater treatment plant (WWTP) in the Greater Sydney area were shown to exhibit significantly reduced gonopodium length (a common biomarker of exposure to estrogenic and/or anti-androgenic EACs) compared with male fish captured at a reference site (Batty and Lim 1999). Follow-up laboratory studies demonstrated significantly decreased gonopodium length and reduced sexual activity after exposure to estrogenic EACs (Doyle and Lim 2002, 2005). Mosquitofish have since been used as bioindicators of EAC exposure in other Australian studies, with varying results. Some studies have reported no significant estrogenic effects in male mosquitofish exposed to domestic secondary treated wastewater but a possible minor androgenic effect resulting in slight elongation of female anal fins (Leusch et al. 2006a), while other studies have found significant reduction in gonopodium length consistent with estrogenic exposure in a lake impacted by urban and agricultural activities (Game et al. 2006).

Endocrine disruption research in Australia has largely focused on WWTP effluent discharges using chemical and, more recently, *in vitro* bioassay methods. As with the mosquitofish studies, those studies have yielded mixed results. Depending on the level of treatment, estrogenic EACs are sometimes detected in WWTP effluent at low ppt concentrations comparable to those found in other developed countries (Table 1). The handful of studies that have analysed river water samples have also detected low concentrations of estrogenic EACs (Chinathamby et al. 2013; Leusch et al. 2010; Tan et al. 2007; Williams et al. 2007; Ying et al. 2009, 2008; Table 1). Whether in Australia or worldwide, most studies to date have measured *in vitro* estrogenic activity and, occasionally, androgenic activity, but almost none have measured anti-estrogenic, anti-androgenic, or (anti)progestagenic activity (GWRC 2012). There is significant cross-talk between endocrine axes, and a recent study indicated that anti-androgens may be as important as estrogens to endocrine disruption in the aquatic environment (Jobling et al. 2009). The level of non-estrogenic endocrine activity in Australian rivers is currently unknown.

To date, the Australian literature on endocrine disruption in the aquatic environment associated with WWTP effluent discharges emphasizes the need for more comprehensive studies (Williams et al. 2007). In particular, there is a need to understand the relative contribution of non-wastewater sources, such as industrial and agricultural inputs. A review on agricultural feedlot wastes (Khan et al. 2008) stressed the need for additional research on dairy shed effluents, which can be a significant source of steroidal input into aquatic environments (Matthiessen et al. 2006). The very few studies in this area suggest that dairy operations may be significant sources of steroid hormones in Australian rivers (Williams et al. 2007; Table 1), and shortened gonopodia in male mosquitofish collected at agricultural and urban sites in Western Australia (Game et al. 2006) suggest that there may well be non-wastewater sources of EACs in Australian rivers.

This study aims to 1) assess multiple classes of EACs by measuring the (anti)estrogenic, (anti)androgenic and (anti)progestagenic activity in Australian rivers using *in vitro* bioassays; 2) identify and quantify known EACs such as steroid hormones and industrial compounds by

trace chemical analysis; and 3) to identify possible trends of EAC concentrations across mainland Australia using the data obtained in the first two objectives, focusing specifically on agricultural, industrial, residential, and wastewater-receiving sites.

114 Table 1. Hormone concentrations and estrogenic and androgenic activity reported in Australian studies. For a review of estrogenic activity in surface waters
115 and effluents in other countries, see Jarosova et al. 2013, Kostich et al. 2013, Loos et al. 2013, Duong et al. 2010, and Jobling et al. 2006. LOD=limit of
116 detection. EEQ = 17β-Estradiol equivalents. TMXEQ = Tamoxifen equivalents. TEQ = Testosterone equivalents. DHTEQ = Dihydrotestosterone equivalents. FluEQ =
117 Flutamide equivalents. LevoEQ = Levonorgestrel equivalents. ACT = Australia Capital Territory. QLD = Queensland. SA = South Australia. NSW = New South Wales. VIC
118 = Victoria. WA = Western Australia.
119

Matrix	Type	State(s)	Chemical results	Bioassay results	Reference(s)
Estrogenic					
Effluent	1 WWTP	QLD	<1 ng/L 17β-estradiol; <1.5 ng/L estrone		Chapman 2003
Effluent	2 WWTPs	NSW	<0.1 - 14 ng/L 17β-estradiol; <0.1 - 54 ng/L estrone; <5 ng/L 17α-ethinylestradiol		Braga et al. 2005a,b
Effluent	1 WWTP	QLD		<0.75 ng/L EEQ (E-SCREEN)	Leusch et al. 2005
Effluent	7 WWTPs	VIC		<LOD - 55 ng/L EEQ (medaka ERBA)	Mispagel et al. 2005
Effluent	15 WWTPs	QLD		<1 - 4.2 ng/L EEQ (ERBA)	Leusch et al. 2006b
Effluent	5 WWTPs (grab samples)	QLD	<LOQ estriol;	1.0 - 67.8 ng/L EEQ (E-	Tan et al. 2007

			<1 - 41.9 ng/L estrone;	SCREEN)	
			<1 - 1.7 ng/L 17α-estradiol;		
			<1 - 1.6 ng/L 17β-estradiol;		
			11.6 - 86.7 ng/L bisphenol A;		
			56.7 - 335.0 ng/L 4-nonylphenol;		
			5.4 - 23.5 ng/L 4-*t*-octylphenol		
Effluent	11 WWTPs	ACT, QLD,	<0.05 - 6.35 ng/L 17β-estradiol;	0.03 - 1.96 ng/L EEQ (YES)	Williams et al. 2007
		SA	3.14 - 39.3 ng/L estrone;		
			<0.05 - 1.30 ng/L 17α-ethinylestradiol;		
			<10 - 148 ng/L bisphenol A;		
			320 - 2,991 ng/L 4-nonylphenol;		
			11 - 165 ng/L 4-*t*-octylphenol		
Effluent	4 WWTPs	NSW		<0.02 - 0.3 ng/L EEQ (YES)	Coleman et al. 2008
Effluent	4 WWTPs	SA	1.0 - 4.2 ng/L 17β-estradiol;		Ying et al. 2008
			13.3 - 39.3 ng/L estrone;		
			0.1 - 1.3 ng/L 17α-ethinylestradiol;		
			12 - 148 ng/L bisphenol A;		
			860 - 2,887 ng/L 4-nonylphenol;		

			12 - 66 ng/L 4-*t*-octylphenol		
Effluent	13 WWTPs	VIC	2 - 18 ng/L 17β-estradiol	1 - 10 ng/L EEQ (Y2H with the hERα)	Mispagel et al. 2009
Effluent	5 WWTPs	QLD	1.37 - 6.35 ng/L 17β-estradiol; 9.12 - 32.22 ng/L estrone; 0.11 - 1.20 ng/L 17α-ethinylestradiol; 13 - 44 ng/L bisphenol A; 614 - 2,991 ng/L 4-nonylphenol; 17 - 165 ng/L 4-t-octylphenol	6.34 - 22.61 ng/L EEQ (calculated using relative potencies derived with the YES assay)	Ying et al. 2009
Effluent	45 WWTPs	VIC	<0.05 - 19 ng/L 17β-estradiol; <0.1 - 18 ng/L estrone; <0.002 - 0.6 ng/L 17α-ethinylestradiol	<0.1 - 73 ng/L EEQ (Y2H)	Allinson et al. 2010
Effluent	2 WWTPs	QLD	<5 ng/L 17β-estradiol; <5 ng/L estriol; <5 - 23 ng/L estrone; <5 ng/L 17α-ethinylestradiol <25 - 50 ng/L bisphenol A;	0.8 - 8 ng/L EEQ (ER-CALUX) 0.14 - 0.15 ng/L EEQ (E-SCREEN)	Leusch et al. 2010

			338 - 1,120 ng/L 4-nonylphenol; 233 - 500 ng/L 4-*t*-octylphenol		
Effluent	9 WWTPs	VIC		<0.1 ng/L EEQ (hERα)	Allinson et al. 2011
Effluent	9 WWTPs	Not specified	<1 ng/L 17α-estradiol; <1 ng/L 17β-estradiol; <5 ng/L estriol; <1 - 20 ng/L estrone; <1 ng/L 17α-ethinylestradiol; <70 - 248 ng/L bisphenol A; <10 - 80 ng/L 4*t*-OP	<0.05 - 5 ng/L EEQ (ER-CALUX)	Leusch et al. 2014a
Effluent	4 WWTPs	WA	<5 ng/ L 17α-estradiol; <5 ng/L 17β-estradiol; <50 - 170 ng/L estriol; <5 – 100 ng/L estrone; <5 ng/L 17α-ethinylestradiol	<0.02 - 6 ng/L EEQ (E-SCREEN)	Leusch et al. 2014b
River	11 Agricultural sites (dairy, grazing, horticulture)	ACT, QLD, SA	0.81 - 3.81 ng/L 17β-estradiol; 1.47 - 13.79 ng/L estrone	0.14 - 1.35 ng/L EEQ (ER-CALUX); <0.3 - 1.22 ng/L EEQ (YES)	Williams et al. 2007

River	2 National parks	SA	0.52 - 4.3 ng/L 17β-estradiol; 0.17 - 4.2 ng/L estrone	0.13 - 0.50 ng/L EEQ (ER-CALUX); <0.3 ng/L ng/L EEQ (YES)	Williams et al. 2007
River	1 Small and 1 large river	QLD	<5 ng/L 17β-estradiol; <5 ng/L estriol; <5 - 7 ng/L estrone; <5 ng/L 17α-ethinylestradiol; <25 ng/L bisphenol A; 221 - 5,270 ng/L 4-nonylphenol; 367 - 483 ng/L 4-*t*-octylphenol	0.15 – 0.5 ng/L EEQ (ER-CALUX); <0.02 - 0.4 ng/L EEQ (E-SCREEN)	Leusch et al. 2010
River	1 Residential	SA	2.9 ng/L 17β-estradiol; 6.1 ng/L estrone	0.19 ng/L EEQ (ER-CALUX); 0.04 ng/L EEQ (YES)	Williams et al. 2007
River	1 WWTP (grab sample)	QLD	<LOQ estriol; 1.5 ng/L estrone; <1 ng/L 17α-estradiol; 7.3 ng/L 17β-estradiol;	<0.1 ng/L EEQ (E-SCREEN)	Tan et al. 2007

			5.5 ng/L bisphenol A; 47.9 ng/L 4-nonylphenol; 1.3 ng/L 4-*t*-octylphenol		
River	5 WWTPs	SA	1.0 - 4.2 ng/L 17β-estradiol; 13.3 - 39.3 ng/L estrone; 0.1 - 1.3 ng/L 17α-ethinylestradiol; 12 - 148 ng/L bisphenol A; 860 - 2,887 ng/L 4-nonylphenol; 12 - 66 ng/L 4-*t*-octylphenol		Ying et al. 2008
River	5 WWTPs	QLD	0.55 - 3.8 ng/L 17β-estradiol; 1.25 - 14.5 ng/L estrone; <LOQ - 0.51 ng/L 17α-ethinylestradiol; 10 - 59 ng/L bisphenol A; 375 - 1,520 ng/L 4-nonylphenol; 18 - 46 ng/L 4-*t*-octylphenol	1.32 - 11.79 ng/L EEQ (calculated using relative potencies derived with the YES assay)	Ying et al. 2009
River	1 WWTP	VIC		<0.3 - 12 ng/L EEQ (Y2H)	Chinathamby et al. 2013
Anti-estrogenic					

Effluent	9 WWTPs	Not specified		<2 µg/L TMXEQ (ER-CALUX)	Leusch et al. 2014a
Androgenic					
Effluent	15 WWTPs	QLD		<6.5 - 736 ng/L TEQ (ARBA)	Leusch et al. 2006b
Effluent	5 WWTPs (grab samples)	QLD	<LOQ - 26.4 ng/L androsterone; <1 - 11.2 ng/L etiocholanolone		Tan et al. 2007
Effluent	45 WWTPs	VIC	<LOD - 14.65 ng/L androstenedione; <LOD - 2.90 ng/L testosterone	<LOD (hAR)	Allinson et al. 2008
Effluent	4 WWTPs	NSW		<0.1 - 8.9 ng/L TEQ (YAS)	Coleman et al. 2008
Effluent	9 WWTPs	Not specified	<5 ng/L dihydrogentestosterione; <5 ng/L testosterone	<2 - 2 ng/L DHTEQ (AR-CALUX)	Leusch et al. 2014a
Effluent	4 WWTPs	WA	<1 - 17 ng/L androstenedione; <50 - 67 ng/L androsterone; <50 ng/L dihydrotestosterone <100 - 180 ng/L etiocholanolone; <1 - 2.4 ng/L testosterone	<2.5 ng/L DHTEQ (AR-CALUX)	Leusch et al. 2014b
River	1 WWTP (grab sample)	QLD	<LOQ androsterone; <1 ng/L etiocholanolone		Tan et al. 2007
Anti-androgenic					

Effluent	9 WWTPs	Not specified	<5 ng/L mestranol	<25 µg/L FluEQ (AR-CALUX)	Leusch et al. 2014a
Progestagenic					
Effluent	9 WWTPs	Not specified	<5 ng/L levonorgestrel	<0.01 - 5.4 ng/L LevoEQ (PR-CALUX)	Leusch et al. 2014a

120

2. Experimental Section

2.1. Chemicals

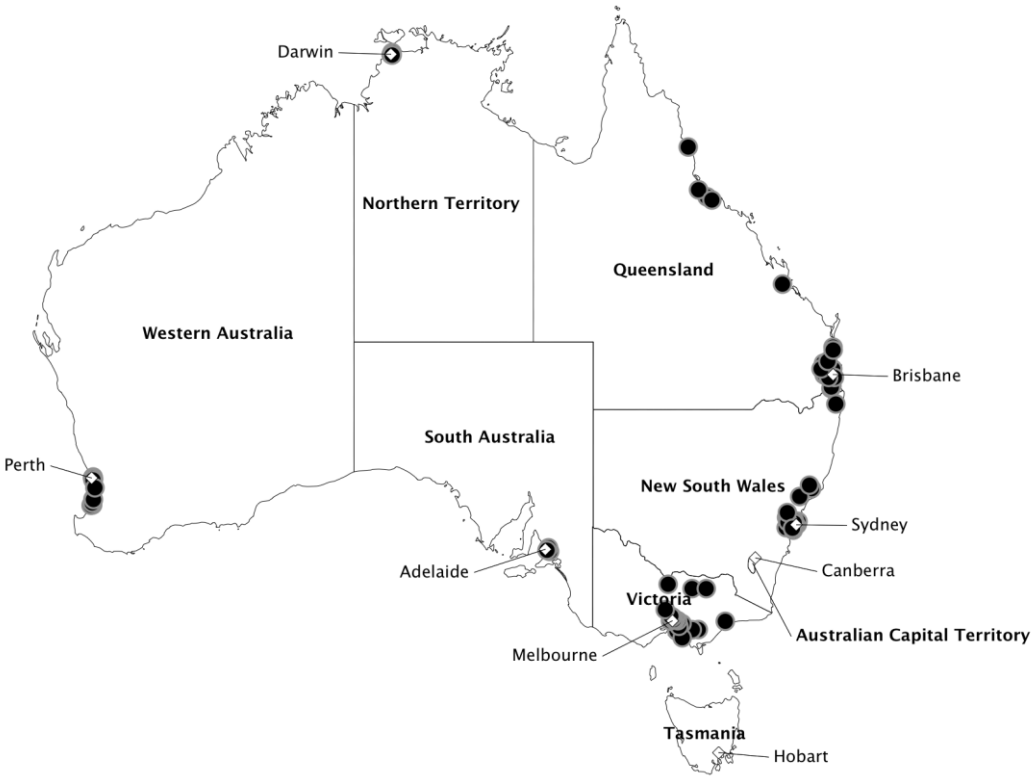
Solvents were analytical reagent grade. Acetone, hexane, methanol and hydrochloric acid (HCl) were provided by Merk (Victoria). Dihydrotestosterone (DHT; >99%), 17β-estradiol (98%), flutamide, levonorgestrel (>99%), mifepristone (>99%), tamoxifen (>99%), ethylenediaminetetraacetic acid (EDTA; >99%) and sodium hydroxide (>98%) were provided by Sigma-Aldrich (New South Wales). All cell culture reagents were purchased from Life Technologies (Victoria). Luciferin was sourced from P.J.K. (Kleinblittersdorf, Germany).

2.2. Site selection

A total of 73 sites were sampled: 19 each in the states of New South Wales (NSW), Queensland (QLD), and Victoria (VIC), 10 in Western Australia (WA), and three each in the Northern Territory (NT) and South Australia (SA) (Fig. 1). Sites were categorized based on the nearest major catchment land-use to the sampling location, but it is important to note that the majority of sample locations were adjacent or downstream of multiple land-use impacts, and simple categorization was generally not possible. Rivers in catchments with agricultural, industrial and residential activity as well as rivers receiving WWTP effluents were chosen, along with sites in catchments with less anthropogenic influences (*i.e.*, "undeveloped" or "reference" sites) from each state/territory (Table 2). Sites were selected following extensive consultation with researchers, regulators and water industry partners familiar with the catchments. The goal was to identify a mix of catchment land-use types in every state.

143 Fig. 1. Location of sampling sites across mainland Australia. State and territory capitals are

144 depicted by white diamonds, while black circles represent sampling locations.



145

146

147 Table 2. Number of samples sorted by land-use and sampling event. A catchment assessment
148 was used to determine the nearest major land-use. WWTP = wastewater treatment plant.
149

	Sample Event			
Nearest major land-use	**Fall (May 2011)**	**Winter (Aug 2011)**	**Spring (Nov 2011)**	**Summer (Feb 2012)**
Agricultural	20	20	20	20
Industrial	7	7	7	7
Residential	19	19	18	18
WWTP	13	13	12	12
Reference	14	14	12	13
Total	**73**	**73**	**69**	**70**

150 *2.3. Water sampling*

151 Samples were collected quarterly from each site (with the exception of NT sites, where
152 samples were collected biannually) over a one-year period. Samples were obtained in autumn
153 (May 2011), winter (Aug 2011), spring (Nov 2011), and summer (Feb 2012) (Table 2). Two
154 acetone-rinsed 1 L amber glass bottles were submerged approximately 30 cm below the water
155 surface in order to collect the 2 L water sample required. To prevent biodegradation, the pH
156 of each water sample was immediately adjusted to approximately pH 2 by addition of 1.5 mL
157 of concentrated HCl (12 M). Basic water chemistry parameters (dissolved oxygen, electric
158 conductivity, pH, temperature) were recorded prior to pH adjustment. Samples were then
159 packaged with frozen ice blocks and sent by overnight courier to the laboratory for solid-
160 phase extraction (SPE). One laboratory blank was created for each sampling event.
161
162 *2.4. Solid-phase extraction*

163 Upon arrival by courier, the pH of the samples was finely adjusted to 2 using HCl (12 M),
164 stored at 4ºC and extracted within 24 h. Samples were vacuum filtered through 2 µm glass
165 fibre filters (47 mm diameter; Millipore, VIC). Each 1 L bottle of sample was passed through
166 a preconditioned SPE cartridge (Waters Oasis HLB SPE cartridges; 500 mg sorbent, 6 cc;
167 Waters, NSW) at 10 mL/min. Conditioning consisted of 10 mL of acetone: hexane (1:1),
168 followed by 10 mL methanol, and finally 10 mL ultrapure water. After passing the full water
169 sample, the SPE cartridges were dried under vacuum at 20 mmHg for 2 h (or until dry). Dried
170 cartridges were wrapped in aluminium foil and stored at 4ºC (for up to 2 weeks) until elution.
171 The polar fraction was eluted with 10 mL methanol and the non-polar fraction was eluted
172 with 10 mL acetone: hexane (1:1). The fractions from replicate cartridges were combined and
173 evaporated under nitrogen until dry and immediately reconstituted in 1 mL methanol for
174 analysis. Reconstituted samples were split into 2 × 500 µL aliquots; one for bioassay analysis
175 and the other for chemical analysis.
176
177 *2.5. Chemical analysis*

178 The industrial compounds bisphenol A and 4-*t*-octylphenol were analysed based on the
179 method of Vanderford and Snyder (2006). Transitions can be found in Table S1. Hormones
180 were analysed by Gas Chromatrography - Tandem Mass Spectrometry (GC-MS/MS) as
181 previously described in Trinh et al. (2011).
182 Standard solutions of all analytes were prepared at 1, 5, 10, 50, 100, 500 and 1000 ng/mL. A
183 relative response ratio of analyte/ internal standard over a 1–1000 ng concentration range was
184 generated enabling quantitation with correction for losses due to ion suppression. All
185 calibration curves had a correlation coefficient of 0.99 or better. Limits of detection (LODs)
186 were defined as the concentration of an analyte giving a signal to noise ratio greater than 3.
187 The limits of quantification (LOQs) were determined using a ratio of greater than 10.
188
189 *2.6. Ethinylestradiol analysis by ELISA*
190 To improve on the GC-MS/MS quantification limit for ethinylestradiol (EE2) of 1 ng/L, a
191 commercially available ELISA (enzyme-linked immunosorbent assay) method was modified
192 to achieve a lower quantification limit. EE2 ELISA kits were purchased from Biosense
193 Laboratories (Norway) and originally sourced from Takiwa Chemical Industries (Japan). In
194 order to decrease the LOQ, standards were first diluted 10× with deionized water, a
195 concentration that still produced a repeatable and concentration-dependent response. Samples
196 were tested twice on different days, and standard curves were generated in duplicate. The
197 LOQ for the full method including sample enrichment by SPE was 0.05 ng/L EE2.
198
199 *2.7. CALUX bioassays*
200 The ERα-, AR- and PR-CALUX bioassays were conducted using previously published
201 methods (Legler et al. 1999; Sonneveld et al. 2011, 2005) with slight modifications noted in
202 Leusch et al. (2010). Luminescence was read in a FLUOstar Omega Spectrometer (BMG
203 Labtech, Germany).
204
205 *2.8. Data analysis and statistics*

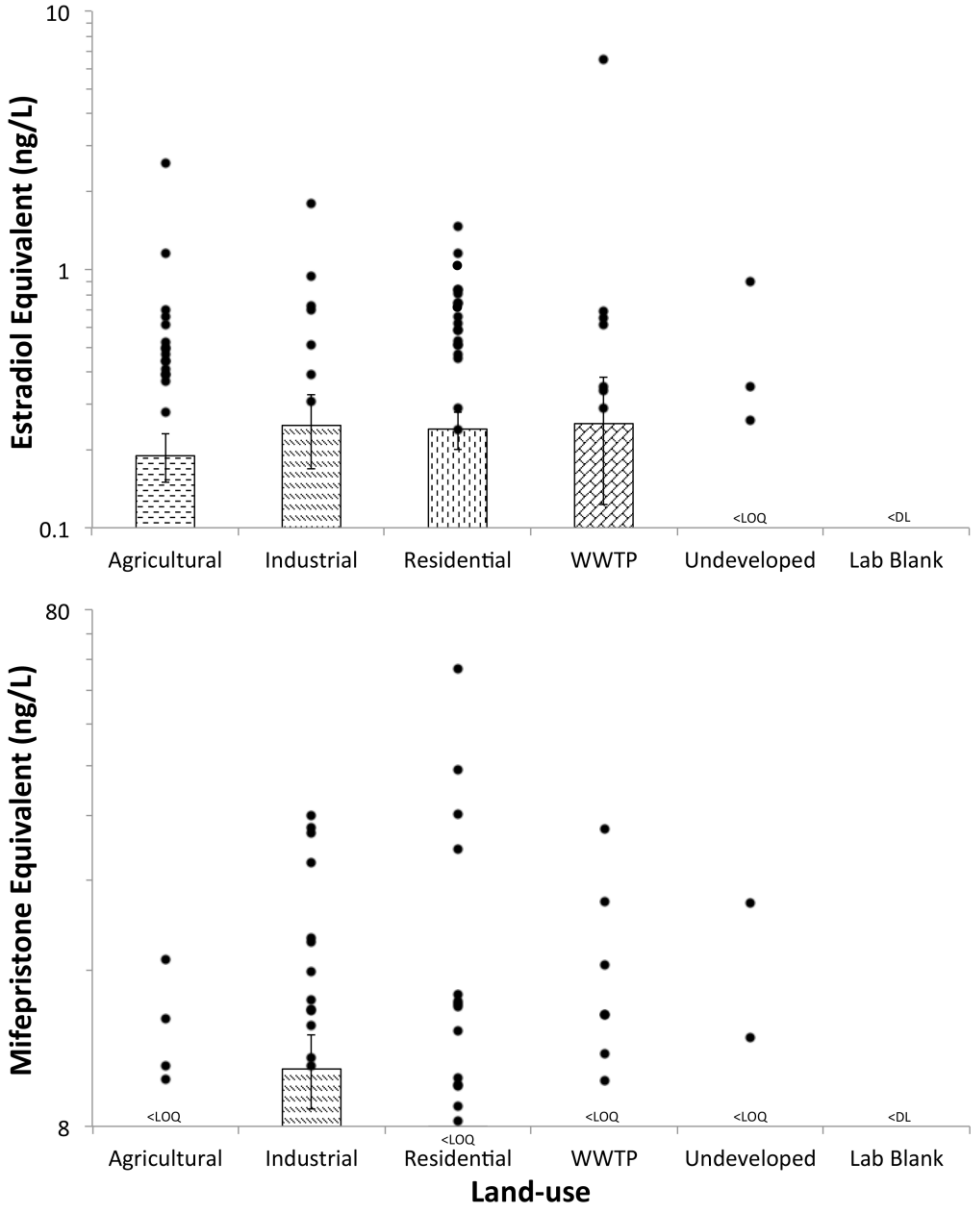
As is often the case with environmental studies, our dataset was heavily left censored with a large proportion of non-detects. Non-detections were assigned a value of half of the LOQ for calculation of averages and standard error of mean (SEM) for graphic and tabular purposes. Kruskal-Wallis nonparametric test followed by Dunn's multiple comparison test ($p<0.05$) were used to determine significant differences in estrogenic and anti-progestagenic activity based on catchment land-use categories. All statistics were performed using Prism 5 software (GraphPad Software, La Jola, CA, USA).

3. Results and Discussion

3.1. Estrogenic and anti-estrogenic activity

Nineteen percent of all samples (53) had quantifiable estrogenic activity (>0.1 ng/L EEQ; Fig. 2 top), with 2% of samples (7) above 1 ng/L EEQ. The detection frequency was highest in residential samples (27%), followed by industrial (25%), agricultural (20%), WWTP (14%) and undeveloped land (6%). The highest estrogenic activity was detected downstream of a WWTP discharge, at 6.5 ng/L EEQ. Anti-estrogenic activity was not detected in any water samples using the ERα-CALUX assay (LOQ = 5 µg/L tamoxifen equivalent, TMXEQ). Our estrogenic results are comparable to those previously obtained in Australia (Table 1) and other industrialized countries. For example, a Dutch study reported 0.2 - 0.5 ng/L EEQ in surface waters using the ERα-CALUX (van der Linden et al. 2008), while a US study calculated a maximum activity of 1.5 ng/L EEQ based on a pharmaceutical assessment and transport evaluation (PhATE) (Anderson et al. 2012). Those concentrations are much lower than what has been reported in a study comparing eight Asian countries, with predicted estrogenic activities of 4 - 46 ng/L EEQ in surface waters based on chemical concentrations (Duong et al. 2010).

Fig. 2. Average estrogenic activity (17β-estradiol equivalent, EEQ) measured with the estrogen receptor CALUX assay (top) and anti-progestagenic activity (mifepristone equivalent, MifEQ) measured with the progesterone receptor CALUX (bottom) of 285 unique extracts from riverine water samples collected quarterly throughout a 1-year period. Water samples separated based on predominant local land-use. Error bars represent the standard error of the mean, with non-detects assigned a value of half the limit of quantification. Points represent values above the mean. "LOQ" = limit of quantification; "DL" = detection limit. * $p<0.05$ (Kruskal-Wallis nonparametric test followed by Dunn's multiple comparison test).



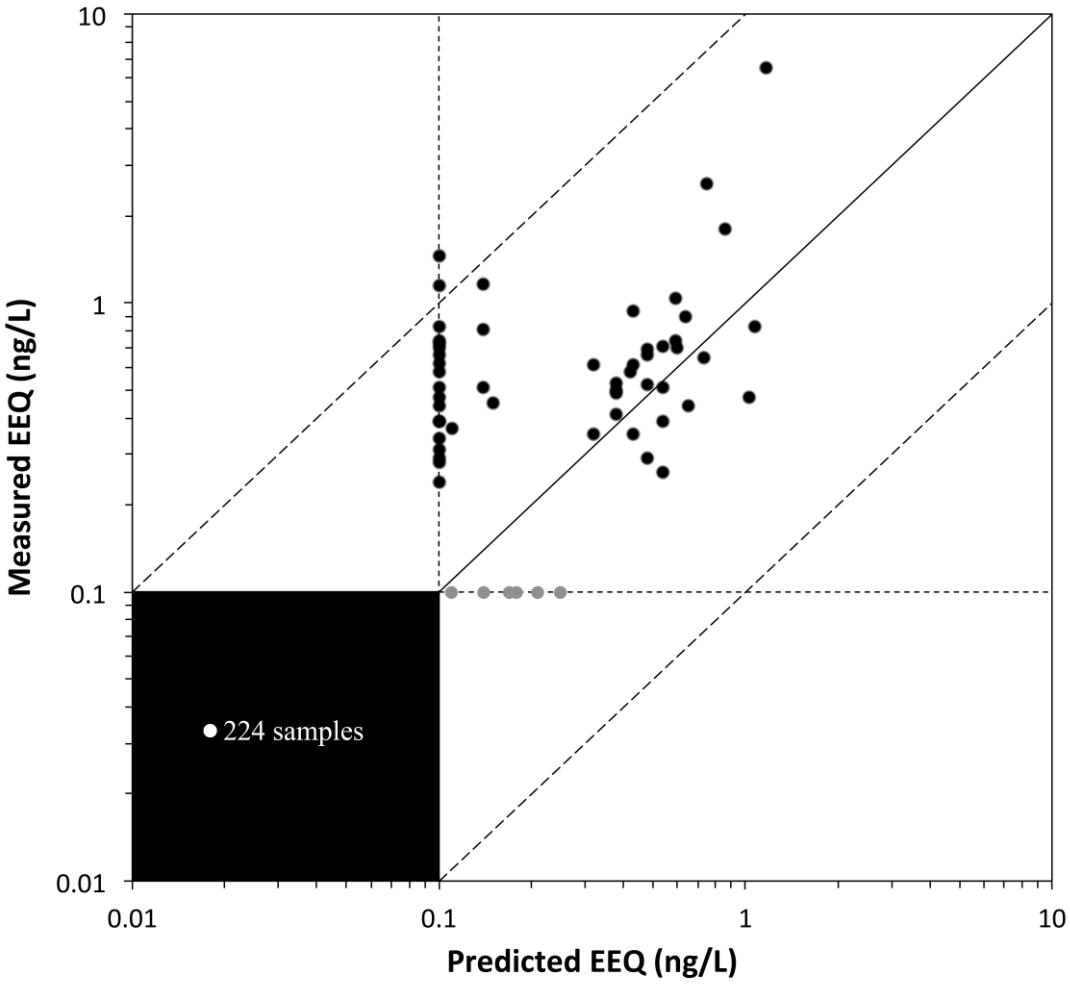
240 There is still some uncertainty as to what constitutes an unsafe level of estrogenic activity in
241 river water. Using a predicted no effect concentration (PNEC) for 17β-estradiol of 1 ng/L
242 (Young et al. 2004) translates into 1 ng/L EEQ in the ERα-CALUX. In our study, seven
243 samples exceeded 1 ng/L EEQ, indicating potential estrogenic effects in exposed aquatic
244 organisms at those sites. None of the samples exceeded 1 ng/L at the same site in subsequent
245 sampling events, which suggests that high estrogenicity was not a persistent condition.
246 However, low-level estrogenicity was detected more than once at each of the seven sites.
247 With one exception, the high estrogenic results occurred in the spring (Nov 2011) and
248 summer (Feb 2012) samples. There was no clear land-use activity associated with these seven
249 high estrogenic results, with three residential, two agricultural, one industrial and one WWTP
250 samples above 1 ng/L EEQ. These results emphasize the need to monitor the aquatic
251 receiving environments more widely and not merely focus on sites downstream of WWTP.
252 Jarosova et al. (2013) derived bioassay-based short-term and long-term safe EEQs (EEQ-
253 SSE) that, when exceeded, indicate a potential *in vivo* hazard. For the ER-CALUX bioassay,
254 the proposed EEQ-SSEs were 0.2 - 0.4 ng/L EEQ for long-term and 0.6 - 2 ng/L EEQ for
255 short-term exposures (Jarosova et al. 2013). All 53 estrogenic samples in this study exceeded
256 the most conservative long-term EEQ-SSE (0.2 ng/L EEQ). In more detail, 18 sites had
257 measurable estrogenicity in only one sample (0.3 - 0.9 ng/L EEQ), while nine sites had two
258 positive samples (0.3 - 6.5 ng/L EEQ). The most conservative short-term EEQ-SSE (0.6 ng/L
259 EEQ) was applied resulting in 19 exceedances at 16 sites; the least conservative short-term
260 EEQ-SSE (2 ng/L EEQ) resulted in only one exceedance. Three sites had estrogenicity in
261 three out of four samples (0.2 - 1.2 ng/L EEQ) and two sites had activity in all four samples
262 (0.3 - 2.6 ng/L EEQ). All of these sites exceeded the long-term EEQ-SSE at least once,
263 regardless of what value was applied. In total, 25 samples were above 0.6 ng/L EEQ and two
264 were above 2 ng/L EEQ.
265 Most of the estrogenic samples in this study had activities between 0.1 and 1 ng/L EEQ. This
266 level of estrogenic activity presents a low risk when compared to the PNEC for 17β-estradiol,
267 but special consideration needs to be given to EE2. Indeed, while EE2 is almost equipotent to

17β-estradiol *in vitro* (Legler et al. 2002), it is 10× more potent *in vivo* and subsequently has a PNEC of 0.1 ng/L (Young et al. 2004; Caldwell et al. 2012). As such, a sample with an EEQ as low as 0.1 ng/L may still be of environmental concern if *all* that activity is caused by EE2 (at 0.1 ng/L). For this reason, all estrogenic samples above 0.1 ng/L EEQ were re-analysed using a more sensitive ELISA method. More than half of all estrogenic samples (28 out of 53) had quantifiable EE2 concentrations (>0.05 ng/L), clearly illustrating that estrogenic activity in river water samples was often associated with the presence of EE2. Almost a quarter (13) of all estrogenic samples had EE2 concentrations above the PNEC of 0.1 ng/L (up to 0.17 ng/L), which is cause for concern, although it should be noted that the sporadic EE2 detections were still at least an order of magnitude lower than the sustained 2-5 ng/L concentration required to cause significant ecological disruption (Kidd et al. 2007), and thus any estrogenic effect is likely to be mild.

To determine the relevance and contribution of the monitored chemicals to the estrogenic activity in the water samples, we compared predicted estrogenicity *vs.* measured estrogenicity in the ERα-CALUX bioassay. Predicted estrogenicity was calculated for each sample as the sum of the product of the concentration of each compound (Table 4) and its potency in the ERα-CALUX assay (Table 3). In most cases, measured estrogenicity was in good agreement with predicted estrogenicity, falling within an order of magnitude of the isometric line (*i.e.*, between the parallel dashed lines on Fig. 3). Only five of the 53 estrogenic samples (measured estrogenicity ≥0.1 ng/L EEQ) were clearly more biologically active than would be expected from chemical analysis alone, indicating either the presence of estrogenic chemicals below the chemical LOQs or unknown estrogenic compounds. There was also good agreement for most (97%) of the 232 samples that did not produce significant estrogenicity in the ERα-CALUX bioassay (data not shown), with measured and predicted estrogenicity in agreement at <0.1 ng/L EEQ. Only eight of those samples had predicted activity higher than 0.1 ng/L EEQ (up to 0.25 ng/L EEQ; grey data points in Fig. 3), with estrone as the only driver of the calculated estrogenicity in all cases. This may indicate the presence of anti-estrogenic

295 compounds in those samples, which would reduce the total estrogenic activity as measured in
296 the bioassay.

297 Fig. 3. Comparison of estrogenic activity (17β-estradiol equivalent; EEQ) measured *in vitro*
298 using the ERα-CALUX assay versus predicted estrogenic activity based on chemical analysis
299 and relative potencies of compounds in the assay (Table 2). Limit of quantification = 0.1 ng/L
300 EEQ. Grey data points were predicted to have estrogenicity, but none was detected. White
301 circle represents samples with neither measured, nor predicted estrogenicity.



302

303 Table 3. Relative potencies of chemicals monitored in this study in the CALUX bioassays. "NA" = Not available; "Neg" = Negative in this assay; "RP" =

304 relative potency. Adapted from Leusch et al. 2014a, except for PR-CALUX antagonistic potencies, and others noted.

	Potency in ERα-CALUX		**Potency in AR-CALUX**		**Potency in PR-CALUX**	
	Agonistic (RP rel to 17β-estradiol; EC_{50} = 1.5E-12M)	Antagonistic (RP rel to tamoxifen; EC_{50} =2.5E-08M)	Agonistic (RP rel to DHT; EC_{50} =3.6E-10M)	Antagonistic (RP rel to flutamide; EC_{50} =9.2E-07M)	Agonistic (RP rel to levonorgestrel; EC_{50} =3.1E-10M)	Antagonistic (RP rel to mifepristone; EC_{50} =2.4E-08M)
Steroids hormones						
Androstenedione	Neg (<0.03)[a]	NA[d]	0.058[a]	NA[d]	Neg (<0.02)[a]	Neg (<3.6E-06)
Androsterone	Neg (<0.03)[a]	NA	0.0056[a]	NA	Neg (<0.02)[a]	Neg (<2.7E-06)
Dihydrotestosterone (DHT)	1.50E-05	Agonist	**1.0**	Agonist	5.20E-06	0.0020
17α-Estradiol (αE2)	0.002	Agonist	Neg (<5.5E-06)	14	Neg (<8.3E-06)	0.0014
17β-Estradiol (βE2)	**1.0**	Agonist	3.98E-05	72	Neg (<6.3E-06)	0.0031
Estriol (E3)	0.017	Agonist	Neg (<6.2E-06)	0.55	Neg (<1.8E-05)	0.0016
Estrone (E1)	0.014	Agonist	Neg (<2.7E-05)	15	Neg (<4.2E-05)	0.0036
17α-Ethinylestradiol (EE2)	5.4	Agonist	Neg (<6.0E-06)	91	4.1E-05	0.0035
Etiocholanolone	NA	NA	NA	NA	NA	0.0021
Levonorgestrel	2.90E-06	Agonist	0.37[b]	NA[b]	**1.0**	Agonist

Mestranol	6.20E-04	Agonist	Neg (<1.6E-05)	2	Neg (<1.9E-05)	9.60E-04
Testosterone	1.70E-06	Agonist	0.17	Agonist	Neg (<1.6E-05)	6.00E-04
Industrial Compounds						
Bisphenol A (BPA)	1.40E-05	Agonist	Neg (<4.6E-06)	0.21	Neg (<7.1E-06)	6.20E-04
4-t-octylphenol (4-*t*-OP)	1.20E-05	Agonist	Neg (<4.2E-06)	0.41	Neg (<1.9E-05)	5.50E-04

305 Notes: [a] Houtman et al. 2009; [b] van den Burg et al. 2010; [c] Sonneveld et al. 2011; [d] Sonneveld et al. 2005.

306 Table 4. Chemical monitoring data of known, detected endocrine active compounds in 285
307 individual water extracts separated by dominant land-use impact at sample location. SEM =
308 standard error of the mean. WWTP=waste water treatment plant.
309

	Count (n)	Detects (n)	Mean ± SEM (ng/L)	Median (ng/L)	95th Percentile (ng/L)	Max (ng/L)
Steroids and Hormones						
Androstenedione						
Agricultural	80	0	<5	<5	<5	<5
Industrial	28	0	<5	<5	<5	<5
Residential	74	1	<5	<5	<5	28
WWTP	50	5	<5	<5	20	36
Undeveloped	53	0	<5	<5	<5	<5
Androsterone						
All land-uses	285	0	<1	<1	<1	<1
Dihydrotestosterone						
All land-uses	285	0	<16	<16	<16	<16
17α-Estradiol						
Agricultural	80	1	<1	<1	<1	4
Industrial	28	0	<1	<1	<1	<1
Residential	74	0	<1	<1	<1	<1
WWTP	50	0	<1	<1	<1	<1
Undeveloped	53	0	<1	<1	<1	<1
17β-Estradiol						
All land-uses	285	0	<1	<1	<1	<1
Estriol						
All land-uses	285	0	<3	<3	<3	<3
Estrone						

Agricultural	80	20	2 ± 0.3	<1	8	18
Industrial	28	4	3 ± 2.0	<1	5	57
Residential	74	24	3 ± 0.8	<1	10	57
WWTP	50	16	2 ± 0.5	<1	7	22
Undeveloped	53	14	1 ± 0.3	<1	3	10
17α-Ethinylestradiol						
Agricultural	80	10	<0.05	<0.05	0.09	0.17
Industrial	28	3	<0.05	<0.05	0.09	0.11
Residential	74	6	<0.05	<0.05	0.06	0.11
WWTP	50	6	<0.05	<0.05	0.09	0.16
Undeveloped	53	3	<0.05	<0.05	<0.05	0.12
Etiocholanolone						
All land-uses	285	0	<6	<6	<6	<6
Levonorgestrel						
All land-uses	285	0	<5	<5	<5	<5
Mestranol						
All land-uses	285	0	<1	<1	<1	<1
Testosterone						
All land-uses	285	0	<5	<5	<5	<5
Industrial Compounds						
Bisphenol A						
Agricultural	80	46	25 ± 3	<20	83	114
Industrial	28	21	308 ± 154	65	1,055	4,130
Residential	74	56	131 ± 34	46	404	1,820
WWTP	50	33	34 ± 7	<20	121	251
Undeveloped	53	31	28 ± 6	<20	84	262
4-t-Octylphenol						
Agricultural	60	2	<10	<10	<10	13
Industrial	21	4	12 ± 4	<10	38	81
Residential	55	11	<10	<10	27	49

WWTP	37	2	<10	<10	<10	11
Undeveloped	39	2	<10	<10	<10	29

310

While there was generally good agreement, predicted estrogenic activity was typically less than the measured activity (in 30 of the 53 estrogenic samples; Fig. 3). Most of those samples (83%) contained the industrial compounds bisphenol A (BPA) or 4-*t*-octylphenol (4-*t*-OP), or both. This hints at the likelihood that other industrial compounds, likely to co-occur with BPA and 4-*t*-OP, are present in concentrations that make up for the discrepancy between the predicted and the (higher) detected estrogenic activity (Fig. 3). One credible candidate is 4-nonylphenol (4-NP); 4-*t*-OP and 4-NP are both long chain alkylphenols, and together with BPA, are used in a variety of industrial operations. 4-NP is also often used as a chemical marker for WWTP impacted water and various isomers of 4-NP are often found in conjunction with 4-*t*-OP (Zhang et al. 2012). Although 4-NP is a potent estrogenic EAC, it has over 550 possible isomers (Zhang et al. 2012) which were not quantified in this study. Often, technical NP (tNP), a mixture of 20-30 NP isomers (Eganhouse et al. 2009; Preuss et al. 2006), is used as a standard for chemical and biological analysis of water samples. However, the biodegradation rate and estrogenic potency of each isomer is dependent on the isomeric composition of the NP molecule, and many isomers are present in impacted freshwater environments (Kim et al. 2005; Routledge and Sumpter 1997). Therefore, using a tNP or a mono-isomeric standard (such as 4-*n*-NP) for chemical and biological analysis will not yield accurate results. 4-*n*-NP was analysed in this study but was not detected in any of the samples, probably due to the rare presence of that specific isomer in environmental mixtures. Given the likelihood of 4-NP being present in water extracts containing industrial compounds such as BPA and 4-*t*-OP, it is probable that a substantial portion of the unaccounted estrogenic activity measured in the 25 samples that contained ether BPA or 4-*t*-OP, or both, is due to 4-NP.

3.2. Progestagenic and anti-progestagenic activity

Progestagenic activity was not detected in any sample (all samples <5 ng/L levonorgestrel equivalent; LevoEQ). However, progestagenic activity was detected recently in Australian WWTP effluents at concentrations up to 5.4 ng/L LevoEQ (Leusch et al. 2014a). In a Dutch

study, an industrial sample (0.96 ng/L LevoEQ) and surface water sample (1.96 ng/L LevoEQ) had higher progestagenic activity compared to the two WWTP effluent samples (0.31 - 0.38 ng/L LevoEQ) (LevoEQ converted from Org2058 equivalents) (van der Linden et al. 2008). A lower detection limit may have detected progestagenic activity that was missed with the LOQ of 5 ng/L LevoEQ in this study. Anti-progestagenic activity (>8 µg/L mifepristone equivalent; MifEQ) was detected in 16% (45) samples. It was detected in more samples from industrial areas (50%) than other land-uses, such as residential (23%), downstream of WWTPs (13%), agricultural (6%) and undeveloped sites (4%) (Fig. 2 bottom). Anti-progestagenic activity was as high as 32 µg/L MifEQ at one industrial river site, however the average activity for all land-uses was <LOQ (8 µg/L MifEQ) due to the large number of samples below LOQ. Similar anti-progestagenic activity was measured in Chinese WWTP effluent (29 µg/L MifEQ, measured with a yeast based bioassay) (Li et al. 2011). The identity of the anti-progestagenic compound(s) is currently unknown. However, 4-NP and BPA at high concentrations have been shown to significantly inhibit the binding of progesterone to recombinant human progesterone receptor (Viswanath et al. 2008). They were also weakly antagonistic in the PR-CALUX assay (Table 3). Many steroid hormones (DHT, 17α-estradiol, 17β-estradiol, estriol, estrone, EE2, etiocholanolone, mestranol and testosterone) also showed weak antagonistic properties in the PR-CALUX. However, for every anti-progestagenic sample (>8 µg/L), there was no predicted anti-progestagenic activity. As such, the causative anti-progestagenic compound(s) responsible for the measured activity is yet to be identified.

BPA was found in 66% of samples across Australia from every land-use category (average = 105 ng/L; Table 4), so further research focusing on its potential anti-progestagenic properties is warranted. The frequency of detection of BPA in this study correlates with other large-scale freshwater studies in the US (31 of 85 samples; 41%) (Kolpin et al. 2002) and The Netherlands (50 of 97 samples; 66%) (Vethaak et al. 2005). Furthermore, BPA, 4-*t*-OP and 4-NP have been found together in a high frequency of samples from WWTP effluents and freshwater environments across the world, such as Switzerland (Ahel et al. 1994), China

(Wang et al. 2013), and France (Cladiere et al. 2013). Therefore, it is probable that some of the anti-progestagenic activity observed in this study is a result of the presence of 4-NP or other alkylphenols we have not been able to identify.

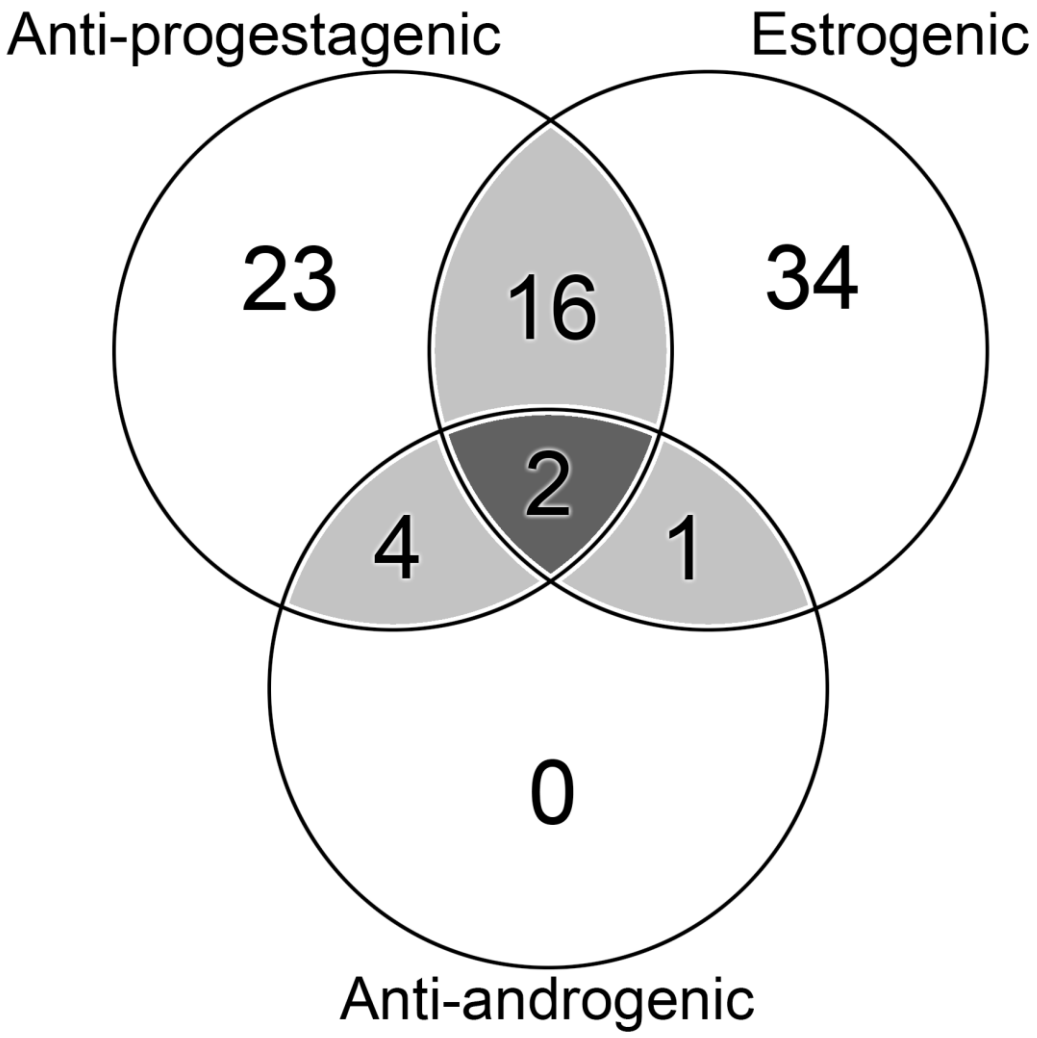
3.3. Androgenic and anti-androgenic activity

Androgenic activity was not detected in any of the samples using the AR-CALUX assay (all <7 ng/L dihydrotestosterone equivalent; DHTEQ), nor was it detected in 45 WWTPs in VIC (Allinson et al. 2008), or 4 WWTP effluents in WA (<2.5 ng/L dihydrotestosterone equivalents; DHT, measured with the AR-CALUX) (Leusch et al. 2014b). Coleman et al. (2008) measured androgenic activity in primary and tertiary WWTP effluents at up to 8.9 ng/L testosterone equivalents (TEQ) using the Yeast Androgen Screen (YAS) assay, while Leusch et al (2006b) measured androgenicity in 15 WWTPs located in QLD with a maximum activity of 736 ng/L testosterone equivalents (TEQ) using the androgen receptor binding assay (ARBA). Another Australian study quantified androgenicity up to 2 ng/L DHTEQ using the AR-CALUX (Leusch et al. 2014a). A Dutch study reported up to 12 ng/L DHTEQ in surface water using the AR-CALUX assay, with the highest concentrations in industrial and hospital wastewater (up to 86 ng/L DHTEQ) and only modest concentrations in WWTP effluents (up to 0.8 ng/L DHTEQ) (van der Linden et al. 2008).

Anti-androgenic activity was only quantifiable in a handful (2%) of samples (>60 µg/L flutamide equivalent; FluEQ). Four residential and 3 industrial samples had anti-androgenic activity ranging from 70 - 257 µg/L FluEQ (data not shown). Predicted anti-androgenic activity based on known potencies (Table 3) and EAC concentrations (Table 4) did not correlate with measured activity, indicating the presence of unknown anti-androgenic EACs. Estrone is a strong anti-androgen in the AR-CALUX, 15× more potent than flutamide (Table 3), but with a maximum concentration of 57 ng/L (Table 4), the anti-androgenic activity due to estrone would still only amount to 0.9 µg/L FluEQ (well short of the LOQ of 60 µg/L FluEQ). This also applies to BPA and 4-*t*-OP. Regardless, their detection most likely

394 indicates the presence of other industrial compounds that may have more potent anti-
395 androgenic properties.
396 Of the seven anti-androgenic samples, six were also anti-progestagenic (Fig. 4). The presence
397 of BPA in each of these samples (62 - 1,820 ng/L) could again correlate with the presence of
398 other unknown industrial compounds with possible endocrine activity, accounting for the
399 remaining anti-androgenic and anti-progestagenic activity.
400
401 *3.4. Samples with multiple endocrine activities*
402 In total, 82 samples produced a biological response in any of the six endpoints measured
403 (ERα+, ERα-, AR+, AR-, PR+ and PR-). The majority of those samples (57 out of 82)
404 produced a biological response in only one endpoint. Twenty-one samples produced a
405 response in two *in vitro* assays, and two samples produced activity in three different assays
406 (Fig. 4). It is well known that certain EACs can affect multiple endocrine functions (*e.g.* BPA;
407 Table 3). Therefore, it is possible that yet-identified EACs having multiple endocrine effects
408 are present, for example in some of the 16 samples that were both estrogenic and anti-
409 progestagenic.

Fig. 4. Ven diagrams depicting samples with quantifiable endocrine activity. ERα-CALUX limit of quantification (LOQ) = 0.1 ng/L 17β-estradiol equivalent (EEQ). Anti-AR-CALUX LOQ = 60 µg/L flutamide equivalent (FluEQ). Anti-PR-CALUX LOQ = 8 µg/L mifepristone equivalent (MifEQ). Anti-estrogenic, progestagenic and androgenic activities were not detected in any samples (LOQ = 5 µg/L tamoxifen equivalent, 0.5 ng/L levonorgestrel equivalent, and 7 ng/L dihydrotestosterone equivalent, respectively).



417 *3.5. Chemical analysis of EACs*

418 Most samples (88%) had a detectable concentration of at least one of the 14 EACs monitored;
419 however, on average, only a handful of the 14 EACs monitored were detected in each sample,
420 with 1.3 compounds detected on average in residential samples, 1.2 in samples downstream of
421 WWTP discharges, 1.1 in industrial samples, and 0.9 each in agricultural and undeveloped
422 samples. The maximum number of EACs detected in one sample was four, in a sample
423 collected downstream of a WWTP.

424 Estrone was the most abundant hormone measured in this study (detected in 27% of samples)
425 with a maximum concentration of 57 ng/L in an industrial sample and a residential sample,
426 both from NSW (Table 4). Estrone was most frequently detected in residential samples and
427 WWTP samples (32% of all samples in each land-use), followed by undeveloped (26%),
428 agricultural (25%) and industrial samples (14%). Estrone detection frequencies did not differ
429 greatly among states but was higher in this study (27%) compared to a US survey (7%)
430 (Kolpin et al. 2002). The concentrations of estrone detected in this study were comparable but
431 slightly higher than most previously reported in Australian rivers and wastewater (Table 1)
432 and other studies (Young et al. 2004), but markedly lower than the maximum concentration of
433 112 ng/L found in a US stream (Kolpin et al. 2002). A PNEC of 6 ng/L for estrone was
434 derived by assessing vitellogenin induction in multiple fish species (Caldwell et al. 2012). Out
435 of the 78 samples that had quantifiable estrone, 24 had concentrations at or above the PNEC
436 indicating a potential hazard to fish at the corresponding sampling locations.

437 EE2 has previously been discussed in some detail (see 3.1). Thirteen samples contained EE2
438 at concentrations greater than its PNEC of 0.1 ng/L (Young et al. 2004; Table 4). Not
439 surprisingly, these concentrations were lower than many Australian studies that measured
440 EE2 in WWTP effluent (Table 1). EE2 concentrations were also lower in this study,
441 compared to river samples taken downstream of WWTPs in other Australian studies (Ying et
442 al. 2009; Ying et al. 2008; Table 1). The maximum EE2 concentration was substantially
443 lower in this study compared to that of a US study (0.17 ng/L *vs.* 831 ng/L), but the detection
444 frequencies were similar (10% and 16%, respectively) (Kolpin et al. 2002). Surprisingly,

445 there was no clear correlation between the presence of EE2 and land-use category: the 13
446 samples with EE2 concentration above 0.1 ng/L were evenly distributed among agricultural
447 (four), residential (three), downstream of WWTPs (two), industrial (two) and undeveloped
448 (two) sites. The existence of the synthetic hormone EE2, primarily used in human birth
449 control pills, in this wide variety of land-use classes indicates the presence of human
450 wastewater even when there is no clear WWTP input, an indication of leaking septic systems,
451 sewage overflow or low-level but widespread wastewater contamination. Considering the
452 very low PNEC for EE2, these results indicate that caution and further investigation is
453 necessary.
454 Of the other estrogen hormones, only 17α-estradiol was detected, and only in one agricultural
455 sample (at 4 ng/L). 17β-Estradiol, estriol and mestranol were not detected above their LOQ (1,
456 3 and 1 ng/L, respectively). While no samples in this study contained 17β-estradiol, a US
457 survey reported its presence in 10% of surface water samples (Kolpin et al. 2002). 17β-
458 Estradiol is frequently detected in Australian wastewater (Table 1), but it has a comparatively
459 short half-life in river water and is degraded to estrone (Jürgens et al. 2002), and thus likely
460 contributes to the wide detection of estrone in our samples.
461 Androgenic hormones were only occasionally detected. A maximum concentration of 36 ng/L
462 for androstenedione was detected twice; once in a SA WWTP sample and once in a WA
463 WWTP sample. Lower concentrations were also detected in three other WWTP samples and
464 one residential sample (Table 4). Androsterone, DHT, etiocholanolone and testosterone were
465 not detected in any sample (n=285) above their LOQ (1, 16, 6 and 5 ng/L, respectively). The
466 only other Australian study measuring androgenic compounds in river samples did not find
467 androsterone or etiocholanolone (Tan et al., 2007; Table 1). Androstenedione, androsterone,
468 etiocholanalone and testosterone have been previously detected in Australian WWTP
469 effluuents at maximum concentrations of 17, 67, 180 and 2.9 ng/L, respectively (Allinson et
470 al. 2008; Leusch et al. 2014b; Table 1).
471 The progestin levonorgestrel was not detected in any sample (LOQ=5 ng/L, n=285).
472 Levonorgestrel, together with EE2, is an active ingredient in the birth control pill and so it is

surprising that it was not detected in this study. Two possible explanations are that it may be metabolized by the body more completely than EE2, or the chemical and *in vitro* LOQs (both 5 ng/L) were simply not sensitive enough to detect levonorgestrel at the environmentally relevant concentrations. High blood plasma concentrations (up to 12 ng/mL) have been found in trout exposed to WWTP effluent (Fick et al. 2010) and concentrations as low as 0.8 ng/L can cause infertility in fathead minnow (Zeilinger et al. 2009). Further investigation using improved method detection limits is certainly warranted considering the occurrence of EE2 in several samples.

With a maximum concentration of 4,130 ng/L in a NSW industrial sample and a detection frequency of 65%, the industrial compound BPA was the most prevalent EAC detected in the present study. BPA was present in 76% of residential samples, 75% of industrial samples, 66% of WWTP samples, and 58% of agricultural samples and undeveloped samples (but was not detected in laboratory blanks). The concentration of BPA reported here was higher than what has previously been reported for Australia (Table 1). BPA was present at a higher frequency in Australian freshwater streams compared to that reported for US streams (65% *vs.* 41%) and had a higher maximum concentration (4,130 ng/L *vs.* 1,900 ng/L) (Kolpin et al. 2002). BPA concentrations were also considerably higher than those reported at a WWTP impacted canal in Greece (380 ng/L) (Arditsoglou and Voutsa 2008) or surface water in Germany (maximum BPA concentration of 410 ng/L) (Fromme et al. 2002). Canada has adopted a predicted no effect concentration (PNEC) of 175 ng/L for BPA (Environment Canada 2008), derived using a 103d exposure and measuring brown trout semen quality and delayed ovulation assay. In our study there were 24 samples with concentrations above 175 ng/L. As such, BPA could be having a negative impact on fish reproduction at the corresponding sample locations.

4-*t*-OP was detected in 21 samples with a maximum concentration of 81 ng/L. It was found frequently in residential samples (20 %). A PNEC of 330 ng/L, established by measuring chronic activity in *Oryzias latipes* (Japanese medaka) (Ministry of the Environment in Japan

2009), indicates that concentrations of 4-*t*-OP reported here are unlikely to be cause for concern.

4-*t*-OP was detected 15 times in the spring sampling event, however it was only detected three times in both winter and summer, and was not detected in autumn. BPA was prevalent in the autumn, winter, and spring, with 47%, 58% and 71% detection frequencies, respectively, but it was only detected in 1% of samples from the summer sampling event. No other temporal trends between sampling events were identified for any of the other monitored chemicals (data not shown).

3.6. Conclusions

Most surface water samples collected in this study (88%) had detectable concentration of at least one of the 14 EACs monitored. Using *in vitro* bioassays, endocrine activity was detected in 28% of samples. Nineteen percent of samples had estrogenic activity >0.1 ng/L EEQ (7 samples exceeding the *in vitro* PNEC of 1 ng/L EEQ), and more than half of those had detectable concentrations of EE2 (0.05-0.17 ng/L), indicating that EE2 often drives the estrogenic response. The PNEC of 0.1 ng/L for EE2 was exceeded in 2% of samples (seven out of 285), indicating a potential hazard to the aquatic environment. Surprisingly, there was no clear association between land-use and EE2 concentration, suggesting either leaking septic tanks, sewage overflow or low-level but widespread wastewater contamination. The conservative short-term EEQ-SSE (0.2 ng/L EEQ) proposed by Jarosava et al. (2013) was exceeded 19 times at 16 estrogenic sites (one to two estrogenic samples out of four). Five sites with consistent estrogenicity (three to four positive samples out of four) exceeded the highest long-term EEQ-SSE proposed (2 ng/L EEQ) at least once. Whether this has caused endocrine effects on the exposed aquatic life is yet unknown.

Anti-progestagenic activity was detected in 16% of all samples; however, the causative compounds were not identified, and environmental implications are unknown. It is currently unknown if the anti-progestagenic activity (<8 - 32 µg/L MifEQ) is severe enough to cause

endocrine disruption in Australian native species, let alone species found throughout the world.

While EACs were detected in most water samples, their concentrations were generally well below levels of concern. Only three compounds were measured above PNEC values: BPA, which was identified in 66% of all samples, exceeded its PNEC of 175 ng/L in 24 samples (8%); estrone, the most frequently detected hormone, exceeded its PNEC of 6 ng/L in 24 samples (8%); and EE2, as previously discussed, exceeded its PNEC of 0.1 ng/L in seven samples (2%). The widespread presence of BPA, and to a lesser extent, 4-*t*-OP, suggests that 4-NP is likely present in many samples, and contributing to measured estrogenic activity, despite not measured due to analytical complications.

Very little information exists on the toxicology of these EACs on Australian native species, but based on the available PNECs it is clear that while the risk is low, endocrine disruption

n may occur intermittently at a small number of sites in Australian rivers.

3.7. Future work

Follow-up studies are warranted to determine the sensitivity to EACs of Australian species, some of which are uniquely different from conventional laboratory test species with which PNECs are often derived. Studies should also investigate anti-progestagenic activity in various waters and identify the compounds responsible for the anti-progestagenic activity detected in surface waters in this study. Further research is also necessary to determine what compounds are contributing to the higher than predicted estrogenic activity, or suppressing the estrogenic activity (as was the case with a few of samples). Finally, while this study suggests there may be intermittent risks to receiving biota at a particularly affected sites, it is unclear what the chronic risk posed by EACs is. The peculiar Australian climate, with alternating periods of flood and drought in particular make this question difficult to answer. The use of passive sampling devices and field assessment of sessile life forms (such as small fish and invertebrates) may provide future insights into this important question.

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