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1 **Assessment of wastewater and recycled water quality: A comparison of lines**

2 **of evidence from *in vitro*, *in vivo* and chemical analysis**

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14

15 **Abstract**

16 Water quality was characterised at an advanced water reclamation plant and three conventional

17 wastewater treatment plants in Southwest Western Australia using a battery of five *in vitro*

18 bioassays. The bioassay battery included endpoints for bacterial toxicity (Microtox), genotoxicity

19 (umuC), photosynthesis inhibition (Max-I-PAM) and endocrine effects (E-SCREEN and AR-CALUX),

20 chosen to provide an appropriately wide coverage of biological effects in the context of managed

aquifer recharge and environmental discharge of treated wastewater. Chemical analysis of hormones and pesticides using LCMSMS was performed in parallel to correlate standard analytical methods with the *in vitro* assessment. For two plants with surface water discharge into drains, further field work was carried out to examine *in situ* effects using mosquitofish (*Gambusia holbrooki*) as a bioindicator species for possible endocrine effects. The results show considerable cytotoxicity, phytotoxicity and estrogenicity in raw sewage, which was significantly reduced by conventional wastewater treatment. No biological response was detected to RO water, suggesting that reverse osmosis is a significant barrier to biologically active compounds. Chemical analysis and *in situ* monitoring revealed trends consistent with the *in vitro* results: chemical analysis confirms the removal trends observed by the bioanalytical tools, and *in situ* sampling did not reveal any evidence of endocrine disruption specifically due to discharge of treated wastewater (although other sources may be present). This study illustrates the utility of combining multiple lines of evidence, including chemical analysis, an *in vitro* toolbox, and *in situ* monitoring in the assessment of water quality.

Keywords: chemical analysis; *in vitro* bioassay; mosquitofish; wastewater; water quality; water recycling

Highlights

- *In vitro* bioassays were combined with chemical analysis and *in situ* sampling.
- Four Western Australian wastewater treatment plants were monitored.
- Slight estrogenic endocrine disruption was detected in receiving environments.
- The three lines of investigation agreed and provided a more complete assessment.
- No biological response or chemicals were detected in reverse osmosis permeate.

1. Introduction

44 There are several methods that can be used to inform environmental risk assessment. Chemical
45 analysis is widely used and well-grounded in regulatory frameworks, but is limited by *a priori*
46 selection of analytes and does not allow assessment of mixture toxicity effects. Assessment of whole
47 animals in the receiving environment (*in situ* analysis) is commonly used to detect toxicants and
48 determine mixture toxicity effects of wastewater, but raises animal ethics issues and is often limited
49 in predictive ability due to complex interactions with environmental factors (such as seasonal
50 variations). *In vitro* bioassays go some way to addressing the limitations of both chemical and *in vivo*
51 analysis and share a mixture of their advantages (*e.g.*, assessment of mixture toxicity, focus on
52 chemical-mediated effects without interference from environmental factors, no ethical issues) and
53 some of their disadvantages (*e.g.*, inability to positively identify causative compounds and accurately
54 predict whole organism effects). Bioanalytical tools also have some unique advantages, including
55 lower detection limits and high throughput screening, and some unique disadvantages such as a lack
56 of regulatory acceptance (Escher and Leusch 2012, Power and Boumphrey 2004). A combination of
57 these different methods would provide valuable additional information to complement the current
58 traditional approach.

59 Climate change has reduced rainfall and significantly diminished water availability over the last 30
60 years in the South West of Australia and further decline is expected over the next 50 years (CSIRO &
61 BOM 2007). At the same time Perth (Western Australia, WA) has an increasing population and
62 competing demands for water. Consequently local authorities are trialling Groundwater
63 Replenishment to supplement aquifers with water reclaimed from urban wastewater at an
64 Advanced Water Reclamation Plant (AWRP). There are also a number of rural wastewater treatment
65 plants (WWTPs) that currently dispose of treated wastewater by discharge to the freshwater
66 environment. The potential for environmental impacts as a result of the ecotoxicity of this effluent is
67 not well understood. This study was undertaken as part of a comprehensive research agenda to

68 better understand the environmental risks associated with managed aquifer recharge (Water
69 Corporation 2013).

70 We investigated the suitability of using three complementary analysis (chemical analysis, *in vitro*
71 bioanalysis and *in situ* biological monitoring) to detect biologically active compounds during
72 wastewater treatment, with a focus on endocrine disruption. We used an "ecotoxicity toolbox" to
73 characterise water quality through the wastewater treatment process. The toolbox includes *in vitro*
74 endpoints for bacterial toxicity (Microtox), genotoxicity (umuC), photosynthesis inhibition (Max-I-
75 PAM) and endocrine effects (E-SCREEN and AR-CALUX). The bioassays were chosen to provide an
76 appropriately wide coverage of biological effects relevant to managed aquifer recharge and
77 environmental discharge of treated wastewater. Chemical analysis using LCMSMS of hormones and
78 pesticides was performed in parallel to correlate standard analytical methods with the *in vitro*
79 assessment. An AWRP in Perth and three WWTPs in neighbouring rural and urban communities were
80 monitored to represent a range of treatment, water quality and end-uses. Samples were taken at
81 various steps in the treatment train in order to characterise water quality through the treatment
82 process. For those plants with surface water discharge into drains (WWTPs 1 and 2), further field
83 work was carried out to examine *in situ* effects using mosquitofish (*Gambusia holbrooki*) as a
84 bioindicator species for possible endocrine effects.

85 The aims of this study were: 1) to analyse water reclaimed by advanced water treatment (including
86 reverse osmosis) intended for managed aquifer recharge with a combination of chemical analysis
87 and *in vitro* bioassays; 2) to analyse raw, partly and fully treated wastewater at two municipal and
88 two rural WWTPs using chemical and *in vitro* methods to determine the quality of representative
89 wastewater in WA; 3) to examine biomarkers of exposure to endocrine disrupting compounds in
90 mosquitofish captured in drains upstream and downstream of two of those WWTPs and analyse
91 water quality at those same sites with the *in vitro* bioassay battery; and 4) to compare the results of

92 the three methods (chemical, *in vitro* and *in situ*) and further our understanding of their value in risk
93 assessment.

94 **2. Materials and methods**

95 *2.1. Site description*

96 Four WWTPs were sampled in this study (Fig 1). The AWRP is a major urban WWTP with a small pilot
97 plant AWRP for trial of groundwater replenishment that includes reverse osmosis (RO) treatment;
98 WWTP 1 and 2 are small rural plants providing secondary treatment of wastewater from country
99 towns with discharge into drains; and WWTP3 is a secondary treatment plant with infiltration to a
100 superficial aquifer with downstream abstraction for irrigation use. The WWTPs were selected to
101 provide a range of treatment technologies and type of end-use/receiving environment typical of
102 Southwest WA.

103 [INSERT FIG 1 ABOUT HERE]

104 Several stages of the treatment trains were sampled (Fig. 1), including raw, partially and fully treated
105 wastewater, and RO permeate at the AWRP. The AWRP was sampled in March, June, September and
106 December 2008. The three WWTPs were sampled in June, September and December 2008 and
107 March 2009. Discharge drains upstream and downstream of WWTP 1 and 2 and a reference site
108 adjacent to the Serpentine National Park were also sampled in December 2008.

109 *2.2. Water sampling and extraction*

110 Quadruplicate samples were taken quarterly over a year at each location: two samples for bioassays,
111 and two samples spiked with deuterated hormone surrogates for chemical analysis.

112 Water samples (1 L for raw sewage, 2 L for all other samples) were collected in methanol-rinsed
113 amber glass bottles by flow-averaged autosampler (AWRP) or by grab samples (all others) and

114 immediately processed. Samples were filtered (AP20 glass fibre filters; Millipore) and extracted by
115 solid-phase extraction using 20 cc Oasis HLB cartridges using the method described in Leusch et al.
116 (2006). In brief, cartridges were pre-conditioned with 10 mL methanol and 10 mL ultrapure water.
117 After passing the entire water sample, the cartridges were air-dried for 2 h. They were eluted with
118 2×10 mL methanol, evaporated to near dryness under a gentle stream of nitrogen, and reconstituted
119 in 500 µL methanol. The eluates were analysed by *in vitro* bioassays and chemical methods. For the
120 first two sampling events (March and June 2008), 10 mL acetone:hexane (1:1) was also used in
121 addition as first conditioning solvent and last elution solvent. In September 2008, side-by-side
122 experiments with wastewater samples showed no significant difference in chemical and
123 bioanalytical results when using acetone:hexane and methanol *vs.* methanol only as conditioning
124 and elution solvent (data not shown), and methanol only was used for the 3 remaining sampling
125 events.

126 *2.3. Bioanalytical toolbox*

127 The Microtox assay measures inhibition of bioluminescence of the naturally-bioluminescent marine
128 bacteria *Vibrio fischeri*. This inhibition is caused by acute cellular toxicity or interference with energy
129 generation pathways, and is responsive to a wide range of toxic chemicals. The assay is used here as
130 representative of non-specific toxicity to bacteria. The assay was adapted from ISO (2007) and
131 performed as described in Escher et al. (2008b). The bioassay threshold (*i.e.*, value above which
132 adverse effects may be expected in the receiving environment) was set to 1 TU (see SI 1.1).

133 The umuC assay measures induction of the SOS response in genetically modified *Salmonella*
134 *typhymurium*. The SOS response is induced in response to single-stranded DNA damage. This
135 bioassay is used here as representative of genotoxicity to bacteria, plants and animals. The assay
136 was adapted from ISO (2000) and performed as described in Macova et al. (2011). The bioassay
137 threshold was set to 1 GTU (see SI 1.2). The umuC assay was run both with and without a liver

138 enzyme fraction (S9), however the data trends were very similar (data not shown) and only the data
139 without metabolic activation are presented here.

140 The Max-I-PAM measures changes in photosynthetic capacity of the algae *Chlorella vulgaris*, and is
141 generally responsive to herbicides. The bioassay is used as representative interference with
142 photosynthesis, a measure of phytotoxicity. The assay was adapted from Schreiber et al. (2007) and
143 performed as described in Escher et al. (2006). The reference compound for this assay was diuron
144 (EC_{50} = 16.8 ± 1.5 µg/L) and the method detection limit was 0.03 µg/L diuron equivalent (DEQ). The
145 bioassay threshold was set at 0.2 µg/L DEQ (see SI 1.3).

146 The E-SCREEN assay measures proliferation of MCF7-BOS breast cancer cells (a gift of Dr Ana Soto,
147 Tufts University), which occurs in the presence of estrogenic compounds such as estrogens and
148 estrogen mimics. The assay is used here as representative of estrogenic endocrine disruption. The
149 assay was adapted from (Soto et al. 1995) and performed as described in (Leusch et al. 2005). The
150 reference compound for this assay was 17β-estradiol (E_2; EC_{50} = 6.5 ± 0.5 ng/L) and the method
151 detection limit was 0.02 ng/L estradiol equivalent (EEQ). The bioassay threshold was set at 0.1 ng/L
152 EEQ (see SI 1.4).

153 Finally the AR-CALUX assay measures induction of an androgenic reporter gene in a genetically-
154 modified human cell line, and is responsive to androgens and androgen mimics. The assay is used
155 here as representative of androgenic endocrine disruption. The assay was adapted from Sonneveld
156 et al. (2005) and performed as described in van der Linden et al. (2008). The reference compound for
157 this assay was 5α-dihydrotestosterone (DHT; EC_{50} = 47.7 ± 3.1 ng/L) and the method detection limit
158 was 2.5 ng/L DHT equivalent (DHTEQ). The bioassay threshold was tentatively set at 10 ng/L DHTEQ
159 (see SI 1.5).

160 *2.4. Chemical analysis*

161 A range of androgen and estrogen hormones were analysed by HPLC-MS/MS analysis as described in
162 Le-Minh et al. (2010). The estrogenic and androgenic hormones analysed (with the limit of reporting)
163 were: the androgens androstenedione (1 ng/L), androsterone (50 ng/L), etiocholanolone (100 ng/L),
164 testosterone (1 ng/L), 5α-dihydrotestosterone (50 ng/L), testosterone propionate (5 ng/L) and 17β-
165 trenbolone (5 ng/L), and the estrogens 17β-estradiol (5 ng/L), 17α-estradiol (5 ng/L), estrone (5
166 ng/L), estriol (50 ng/L) and 17α-ethinylestradiol (5 ng/L).

167 Herbicides analysis was performed at Queensland Health Forensic and Scientific Services (QHFSS).
168 The following pesticides were measured: flumeturon, diuron, simazine, atrazine, desethyl atrazine,
169 desisopropyl atrazine, hexazinone, tebuthiuron, ametryn, prometryn, bromacil, terbutryn,
170 metolachlor and imidacloprid. The reporting limit for all compounds was 0.025 µg/L. The compounds
171 were analysed by HPLC-MS/MS using an AB/Sciex API300 mass spectrometer (AB/Sciex Concord,
172 Ontario, Canada) equipped with an electrospray (TurboSpray) interface coupled to a Shimadzu
173 LC10AD HPLC system (Shimadzu Corp., Kyoto, Japan). Separation was achieved using a 5 µm 150×3
174 mm Aquastar column (Thermo Scientific, Australia) run at 40 ºC, and a flow rate of 0.6 mL/min with
175 a linear gradient starting at 10% B for 1 min, ramped to 100% B in 6 min, held for 2.5 min and then
176 set to 10% B in 15 sec and equilibrated for 4 minutes (A = 10% methanol/HPLC grade water, B = 90%
177 methanol/HPLC grade water, both containing 5 mM ammonium acetate). The mass spectrometer
178 was operated in positive ion multiple reaction-monitoring mode using nitrogen as the collision gas.
179 Positive samples were confirmed by retention time and by comparing transition intensity ratios
180 between the sample and an appropriate concentration standard from the same run. Samples were
181 reported as positive if the two transitions were present, retention time was within 9 sec of the
182 standard and the relative intensity of the confirmation transition was within 20% of the expected
183 value.

184 *2.5. Mosquitofish sampling and analysis*

185 Mosquitofish were collected in September 2008, March and July 2009 (Spring, Summer and Winter)
186 immediately downstream of the discharge point of WWTP 1 and 2. For each WWTP an upstream site
187 was selected for sampling with considerations of low potential of population mixing between the
188 two sites on the same stream. Fish were also collected in the Serpentine River at a site adjacent to
189 Serpentine National Park (reference site).

190 On each sampling occasion up to 100 individuals were collected until the catch per unit effort
191 became too low to justify further capture. Twenty adult males (identified by the presence of an
192 elongated anal fin) and 6 females (identified by a black spot on the ventral side of the abdomen)
193 were snap-frozen in liquid nitrogen. The remainder were transported on ice to the laboratory.

194 Fish were categorised as adult male, immature male, adult female and immature female. Sex and
195 stage of maturation based on anal fin morphology was determined using the methods described by
196 Rawson et al. (2009). Morphometric measurements including gonopodial length (GL), pre-anal
197 length (PAL), 6th ray length (based on Game et al. 2006), standard length and wet weight were made
198 on mature and immature male fish.

199 Vitellogenin was quantified in fish taken from each site in Summer (March 2009) and Winter (July
200 2009). Where possible 20 male and 6 female fish from each sampling site were homogenised in 1:4
201 (w:v) Tris buffer (3 mM Tris, 0.1 µM PMSF). The homogenates were centrifuged at 8000×*g* for 40
202 minutes at 4 ºC. Homogenates and a salmonid vitellogenin standard (Biosense Laboratories, Bergen,
203 Norway) were diluted (1:1000) in a carbonate/bicarbonate buffer (50 mM, pH 9.6). Samples and
204 standard were added to wells of a clear 96-well plate and allowed to incubate for 24 h at 4 ºC. The
205 plates were then washed with phosphate buffered saline (0.05 % Tween). A 1% BSA blocking
206 solution was added to each well and, following 1 h incubation, the monoclonal antibody BN-5
207 (Biosense Laboratories) was added and incubated for 1 h at 37 ºC. The plates were again washed
208 (3×) and a goat anti-rabbit HRP conjugate (Biosense Laboratories) was added for a further 1 h

209 incubation at room temperature. After 5 wash cycles a peroxidase substrate (OPD fast; Sigma) was
210 added to the wells for a 15 min dark incubation. The reaction was stopped with 2 M H_2SO_4 and the
211 absorbance read immediately at 490 nm.

212 *2.6. Data analysis*

213 *2.6.1. Bioanalytical data analysis*

214 Biological response in water samples was quantified by calculating their EC_{50} in each bioassay,
215 expressed in units of Relative Enrichment Factor REF (calculated by dividing the sample
216 concentration from solid phase extraction by the dilution in the assay, as described in Macova et al.
217 (2011)).

218 Microtox results are expressed as Toxic Unit (TU), calculated as $1/EC_{50(REF)}$ of the sample; umuC
219 results are expressed as genotoxic unit (GTU), calculated as $1/EC_{50(REF)}$. Max-I-PAM, E-SCREEN and
220 AR-CALUX results are expressed as bio-equivalents, calculated as EC_{50} of the reference compound
221 (diuron, 17β-estradiol and 5α-dihydrotesterone, respectively) divided by $EC_{50(REF)}$ of the sample.

222 *2.6.2. Predicted biological response*

223 *In vitro* responses could be predicted for estrogenicity, androgenicity and photosynthesis inhibition
224 by multiplying the concentration of selected compounds by their relative potency (RP; determined in
225 this study) in the relevant assay. Cytotoxicity and genotoxicity can be caused by a wide variety of
226 compounds, and it was therefore not possible to relate those biological endpoints to chemical
227 analysis.

228 Predicted estrogenicity was calculated from 17β-estradiol (RP = 1), estrone (RP = 0.012) and estriol
229 (RP = 0.071); predicted androgenicity was calculated from androstenedione (RP = 0.057),

230 androsterone (RP = 0.006), testosterone (RP = 0.21), testosterone propionate (RP = 0.20) and 17β-

231 trenbolone (RP = 0.50); photosynthesis inhibition was calculated from diuron (RP = 1).

232 *2.6.3. Morphological measures*

233 For *in situ* measurements, differences in GL and PAL were analysed using single factor analysis of

234 covariance (ANCOVA) with the length of the fish as a covariate. The assumptions of homogeneous

235 covariate slopes and equal variances between groups were satisfied. Sex ratios were examined using

236 a binomial test.

237 **3. Results and discussion**

238 *3.1. Bioanalytical*

239 In general, raw sewage caused high *in vitro* responses, however wastewater treatment effectively

240 removed most of the activity and the treated wastewater samples were generally below bioassay

241 threshold values (Fig. 2). At the AWRP, the aerobic and anaerobic digestion (secondary treatment)

242 and reverse osmosis provided most of the removal, while the oxidation ditch at WWTP3 performed

243 generally well (although it created short-lived genotoxicity). The raw sewage at the rural WWTPs

244 (WWTP 1 and 2) was much less toxic than at the municipal plants (AWRP and WWTP3), and the

245 pond-based treatment systems were adequate at reducing the toxicity to levels comparable to

246 AWRP secondary-treated wastewater and WWTP3 effluent. The site downstream of WWTP2

247 discharge induced slightly higher biological response *in vitro* compared with the upstream site,

248 although it was usually below bioassay threshold levels.

249 [INSERT FIG 2 ABOUT HERE]

250 *3.1.1. Microtox assay for bacterial toxicity*

251 At the municipal plants (AWRP and WWTP3), raw sewage was acutely toxic to bacteria but aerobic
252 and anaerobic digestion (at AWRP) and the oxidation ditch (at WWTP3) reduced the toxicity of the
253 water by 89-92% and 70-91%, respectively. This suggests that toxic chemicals are either mineralised
254 to less toxic degradation products or absorbed to sludge particles and removed from the water
255 stream. At the rural plants (WWTP 1 and 2), raw sewage was not particularly toxic to bacteria (<0.3
256 TU) and the effectiveness of pond-based treatment was variable. The treated wastewater samples at
257 all plants monitored in this study were below 1 TU, indicating that only minimal acute toxicity would
258 occur at environmental levels. Discharge of treated wastewater at WWTP 1 and 2 resulted in a 3-5×
259 increase in baseline toxicity in the drain (Fig. 2, top right), but the response was still significantly less
260 than 1 TU. The Microtox assay is sensitive to a wide range of compounds, with a strong correlation
261 between "toxicity" and lipophilicity (Tang et al. 2013). The responses with environmental water
262 samples (Fig 2, Microtox, Rivers) therefore indicates the presence of a variety of compounds, but at
263 concentrations that are not acutely toxic to bacteria (TU < 1).

264 RO treatment at AWRP reduced cytotoxicity of the water stream by a further 68% at least. Low level
265 responses (<0.05 TU) were detectable in 2 out of 4 RO permeate samples and the water at the
266 reference site (Fig 2, Microtox, "RO" and "Ref"). Low level response in highly concentrated water
267 samples (REF > 10) with non-specific assays such as the Microtox has been reported previously
268 (Escher et al. 2008a) and is thought to be an artefact of solvent carry-over during the SPE
269 concentration step. It is of no environmental concern as long as a) it is not considerably above the
270 detection limit (*i.e.*, less than 0.1 TU) and b) no other specific and/or reactive toxicity are associated
271 with the sample – as was the case here.

272 These results are comparable to previous Australian studies, which reported the equivalent of 0.20
273 TU in raw sewage, up to 0.10 TU in secondary treated wastewater, and up to 0.04 TU in tertiary
274 treated wastewater using the Microtox assay (Macova et al. 2011, Muller et al. 2007).

275 *3.1.2. umuC assay for genotoxicity*

276 Raw sewage contained detectable levels of genotoxicity (Fig. 2, umuC -S9). Aerobic and anaerobic
277 digestion at AWRP resulted in a 15-44% reduction in genotoxicity. Oxidation at WWTP3 resulted in a
278 4.9-6.2× increase in genotoxicity to levels above 1 GTU. An increase in genotoxicity in such an
279 environment favouring the creation of reactive compounds is not unexpected, but it should be noted
280 that it was short lived and that genotoxicity dropped below the bioassay threshold after clarification.
281 The genotoxicity of the final wastewater was 60-75% lower than that of the raw influent, and was
282 similar to that of the secondary wastewater at the AWRP. Genotoxicity was not commonly detected
283 in rural wastewater (WWTP 1 and 2) and was not detected in any of the river water samples.

284 Reverse osmosis was the most effective removal method for genotoxicity, removing at least 86% of
285 the activity (to <0.02 TU). Genotoxicity was not detected in any of the RO permeate samples (Fig. 2).

286 With the exception of samples from the oxidation ditch, all samples were below 1 GTU. Their
287 discharge into the environment would therefore not be expected to produce significant genotoxicity.
288 The samples from the oxidation ditch, however, were above 1 GTU, suggesting that if this water
289 were discharged without further treatment (which is not the case) it may cause adverse effect in
290 exposed microorganisms.

291 These results are comparable to previous Australian studies, which reported the equivalent of 0.4
292 GTU in raw sewage and less than 0.02 GTU in treated wastewater using the umuC -S9 assay (Macova
293 et al. 2011, Muller et al. 2007).

294 *3.1.3. Max-I-PAM assay for photosynthesis inhibition*

295 Raw sewage at the municipal plants (AWRP and WWTP3) strongly inhibited photosynthesis, with the
296 phytotoxicity above the bioassay threshold of 0.2 µg/L DEQ in most samples (Fig. 2, Max-I-PAM).
297 Aerobic/anaerobic digestion (at AWRP) and oxidation ditch (at WWTP3) reduced phytotoxicity by 48-

298 90% and 50-86%, respectively. Reverse osmosis removed a further 48% at least, and all RO permeate
299 samples were below quantification limit (<0.03 μg/L DEQ).

300 The phytotoxicity in rural raw sewage (WWTP 1 and 2) was similar, ranging from 0.04-0.23 μg/L DEQ.
301 The overall removal efficacy with the pond-based systems was 47-59%, on average. The
302 phytotoxicity in the treated wastewater was similar to that of the municipal WWTPs, ranging from
303 <0.03-0.08 μg/L DEQ. Low-level photosynthesis inhibition was detected downstream of WWTP2
304 discharge (0.06 μg/L DEQ), but not at WWTP1. All treated wastewater samples were below the
305 bioassay threshold of 0.2 μg/L DEQ (Fig. 2).

306 These results are comparable to previous Australian studies, which reported up to 0.22 μg/L DEQ in
307 raw sewage, 0.05-0.28 μg/L DEQ in treated wastewater, and up to 0.04 μg/L DEQ in river water
308 (Escher et al. 2006, Macova et al. 2011, Muller et al. 2007). Similar concentrations have been
309 reported in Switzerland, with an average of 0.28 μg/L DEQ in raw sewage, 0.19 μg/L DEQ in treated
310 wastewater, and up to 0.23 μg/L DEQ in river water (Escher et al. 2008b).

311 *3.1.4. E-SCREEN assay for estrogenic EDCs*

312 Raw sewage at the municipal plants (AWRP and WWTP3) was highly estrogenic (Fig. 2, E-SCREEN).
313 Aerobic and anaerobic digestion (at AWRP) reduced estrogenicity by 82-98%, consistent with
314 expectations that estrogens would mineralise and/or absorb to sludge (Andersen et al. 2003). The
315 estrogenicity in the secondary treated wastewater from the municipal treatment plants (AWRP and
316 WWTP3) was still several orders of magnitude higher than a bioassay based threshold of 0.1 ng/L
317 EEQ, suggesting that release of these wastewaters without dilution could cause estrogenic effects in
318 the receiving environment.

319 Reverse osmosis removed at least 39% of the remaining estrogenicity, and all RO permeate samples
320 were below detection limit (<0.02 ng/L EEQ).

321 Estrogenicity at the rural WWTPs (WWTP 1 and 2) was very low, with less than 4.8 ng/L EEQ in raw
322 sewage and <0.02 ng/L EEQ at all other stages. Only river samples upstream and downstream of
323 WWTP2 had detectable estrogenicity, with a 2.2× increase at the downstream site (up to the
324 bioassay threshold of 0.1 ng/L; Fig. 2, E-SCREEN, "DN2"). This level of estrogenicity suggests that low
325 level estrogenic endocrine effects may be detected in fish collected from that site (see Sections 3.3
326 and 3.4.2), as well as the existence of upstream sources of estrogenic compounds.

327 The concentrations are consistent with previous Australian studies, which reported up to 74 ng/L
328 EEQ in raw sewage, up to 1.6 ng/L EEQ in secondary treated wastewater, up to 0.47 ng/L EEQ in river
329 water, and less than 0.06 ng/L EEQ in reclaimed water in the E-SCREEN assay (Leusch et al. 2010,
330 Macova et al. 2011, Tan et al. 2007).

331 *3.1.5. AR-CALUX assay for androgenic EDCs*

332 Raw sewage contained high levels of androgenic compounds, but wastewater treatment was
333 effective at reducing the androgenicity in all treated wastewater samples to below detection limit
334 (<2.5 ng/L DHTEQ). None of the environmental samples had any detectable level of androgenicity,
335 suggesting that androgenic endocrine disruption is unlikely in organisms in the receiving
336 environment. All RO permeate samples were also below detection limit.

337 No data could be found on androgenicity in Australian waters, but our results are comparable with a
338 European study, which reported up to 86 ng/L DHTEQ in raw sewage and 0.75-0.83 ng/L DHTEQ in
339 treated wastewater (van der Linden et al. 2008).

340 *3.2. Chemical*

341 *3.2.1. Androgens and estrogens*

342 Several androgen and estrogen hormones were detected in raw and partially treated wastewater
343 (Table 1). Overall, the chemical data indicates good removal by secondary treatment and, with a few
344 exceptions, hormones were not detected in treated wastewater. Several androgens were routinely
345 detected (particularly the naturally occurring androstenedione, androsterone, etiocholanolone and
346 testosterone). Estrone was the only estrogen commonly detected, including in secondary treated
347 wastewater (Table 1). Three hormones (dihydrotestosterone, 17α-estradiol and 17α-
348 ethynylestradiol) were not detected in any of the samples (reporting limits of 50, 5 and 5 ng/L,
349 respectively). None of the monitored chemicals were detected in RO permeate.

350 [INSERT TABLE 1 ABOUT HERE]

351 The concentrations and observed removal during treatment at the four WWTPs investigated in this
352 study are consistent with previous reports in Australian wastewater (Allinson et al. 2010). The only
353 unusual finding was the observation of the androgenic steroid 17β-trenbolone in raw influents and
354 primary treated wastewater (although it was not observed in secondary or more highly treated
355 wastewater). 17β-Trenbolone is the metabolite of the hormone growth promoter 17β-trenbolone
356 acetate used in beef cattle, suggesting that agricultural run-off may be the source of this chemical.
357 The trenbolone concentrations detected here were in the same range as those previously reported
358 in runoff from beef feedlot in the USA (Durhan et al. 2006).

359 *3.2.3.Herbicides*

360 Of all the pesticides monitored (section 2.2.2), only diuron and metolachlor were detected above the
361 reporting limit (0.025 µg/L).

362 Diuron was detected at most WWTPs, at concentrations up to 0.15 µg/L. Conventional wastewater
363 treatment was not particularly effective, and concentrations of diuron in treated wastewaters were
364 in the same range as in the influent. Reverse osmosis was, however, effective at removing diuron to

365 below quantification limit. Metolachlor was detected at the AWRP only, at up to 0.12 µg/L in raw
366 sewage. Primary and secondary treatment removed more than 78% of the compound.

367 *3.3. Mosquitofish*

368 There were significant differences between sex ratios at both WWTPs in the September (Spring) and
369 March (Summer) sampling periods but not during the July (Winter) sampling period (Table 2).
370 However, the trends were not consistent between sampling periods and appear to reflect
371 population differences rather than any WWTP impact.

372 [INSERT TABLE 2 ABOUT HERE]

373 There was no consistent effect on GL or PAL at either WWTP 1 or 2. There were no significant
374 differences between either the GL (ANCOVA, $p_{Sep} = 0.214$, $p_{Jul} = 0.472$) or PAL (ANCOVA, $p_{Sep} = 0.546$,
375 $p_{Jul} = 0.091$) of fish captured upstream and downstream of WWTP1 in September 2008 or July 2009.
376 Fish from upstream and downstream of WWTP1 in March 2009 had similar GLs (ANCOVA, $p = 0.083$).
377 In March 2009 male fish had significantly shorter PALs downstream of WWTP1 compared to fish at
378 the upstream site (ANCOVA, $p = 0.038$). While this potentially represents an androgenic effect, it was
379 not repeated in any of the other endpoints at any other of the sampling periods. There was also no
380 significant difference between the upstream and downstream sites at WWTP2 in terms of GL
381 (ANCOVA, $p_{Sep} = 0.112$, $p_{Mar} = 0.323$, $p_{Jul} = 0.472$) or PAL (ANCOVA, $p_{Sep} = 0.104$, $p_{Mar} = 0.324$, $p_{Jul} =$
382 0.091). However, when compared to fish from the reference site, fish from downstream of WWTP2
383 had longer gonopodia (ANCOVA, $p = 0.001$) and longer PAL (ANCOVA, $p < 0.001$).

384 Vitellogenin (Vtg), a precursor to egg yolk, is naturally produced by mature female fish in the lead up
385 to the reproductive season (Summer). Vtg was not detectable in plasma of female fish in Winter (Fig.
386 3, right, grey bars) but commonly detected in females in Summer (except at the reference site; Fig. 3,
387 right, black bars). Vtg is not normally produced in male fish except upon exposure to estrogenic

388 compounds (Sumpter and Jobling 1995). If males were exposed to estrogenic compounds, it could be
389 expected that Vtg would be produced irrespective of the season. Plasma Vtg was not detected in
390 male mosquitofish at WWTP1 and the reference site (Fig. 3, left); however, it was detected in both
391 Summer and Winter sampling periods upstream and downstream of WWTP2 (at low levels
392 compared to female fish). This indicates that these fish were exposed to estrogenic compounds.
393 Given that fish both upstream and downstream of the WWTP outlet displayed this response, it is not
394 possible to attribute this to the presence of the outlet alone. It is likely that upstream sources of
395 EDCs are acting, with the outlet contributing to the EDC load at the downstream site.

396 [INSERT FIG 3 ABOUT HERE]

397 *3.4. Comparison of the different methods*

398 *3.4.1. Comparison of chemical and in vitro bioassay analysis*

399 Using the concentrations of chemicals and their known potency in the bioassays, it was possible to
400 calculate predicted activity and compare it with the measured response *in vitro*. This could be done
401 for estrogenicity based on estrogen hormones, androgenicity based on androgen hormones and
402 photosynthesis inhibition based on diuron (Fig 4).

403 [INSERT FIG 4 ABOUT HERE]

404 The natural hormones 17β-estradiol and estriol accounted for most of the predicted estrogenicity in
405 raw and partially treated wastewater (92-94%), while estriol and estrone accounted for most of the
406 predicted estrogenicity in secondary treated wastewater (90%).

407 When comparing predicted with measured estrogenicity, 30 out of 66 data points (45%) were within
408 an order of magnitude from isometric agreement (*i.e.*, within the dotted lines in Fig 4). Out of the 36
409 data points that were more than one order of magnitude away, 26 predicted much higher

410 estrogenicity than was measured (24 of those at WWTP 1 and 2) and 10 measured much higher
411 estrogenicity than predicted. The chemical detection limit for the potent estrogen 17α-
412 ethinylestradiol was comparatively high (5 ng/L) and it is likely that this compound occurring just
413 below the chemical detection limit explains most of the occurrences of measured higher than
414 predicted estrogenicity. The presence of anti-estrogens may explain the poor agreement between
415 predicted and measured estrogenicity was found at the rural WWTPs 1 and 2.

416 Testosterone, androstenedione and 17β-trenbolone were evenly responsible for 83-93% on average
417 of the predicted androgenicity in raw and primary treated wastewater, while androstenedione alone
418 was responsible for 67-78% of the predicted androgenicity in partially treated and secondary treated
419 wastewater.

420 Much better agreement between predicted and measured response was obtained for androgenicity,
421 with all 62 data points falling within an order of magnitude of isometric agreement (Fig 2). This
422 suggests that the 5 androgens measured here by chemical analysis are responsible for most of the
423 androgenicity in the AR-CALUX assay.

424 When comparing predicted and measured photosynthesis inhibition, there was generally good
425 agreement between the predicted and measured diuron equivalent (with 67 out of 83 data points
426 within an order of magnitude of isometric agreement, Fig 4). There were no samples where the
427 response was lower than predicted, however the measured response was much higher than
428 predicted for 19% of samples. This is particularly true for raw sewage samples, and suggests the
429 presence of other phytotoxic compounds not accounted for by chemical analysis. Note that
430 measured and predicted photosynthetic inhibition in all secondary and treated wastewater samples
431 were in good agreement, indicating that diuron was the most significant contributor to phytotoxicity
432 in these samples. This suggests that phytotoxic compounds present in raw sewage were removed by
433 wastewater treatment, but that diuron was more persistent.

434 *3.4.2. Comparison of* in situ *monitoring and analysis of wastewater at WWTP 1 and 2*

435 There was a good parallel between the findings of the *in situ* monitoring and both chemical and *in*
436 *vitro* bioassay analysis of the treated wastewaters and rivers. Overall, the chemical and *in vitro*
437 bioassay analysis showed good removal of endocrine-active substances (both estrogenic and
438 androgenic) by wastewater treatment to produce low to undetectable concentrations of estrogenic
439 and androgenic EDCs (Table 1 and Fig 2). Low estrogenicity was detected in the drain upstream and
440 downstream of WWTP2, with the downstream site slightly higher than the upstream site (Fig 2, E-
441 SCREEN). Based on these findings, slight estrogenic effects were expected in fish in the WWTP2 drain
442 but not in the WWTP1 drain or at the reference site. This is indeed what was found (Fig 3).

443 All three lines of evidence (chemical analysis, *in vitro* bioassay analysis and *in situ* whole fish
444 monitoring) suggest that treatment at the rural WWTPs monitored in this study is adequate to
445 remove EDCs present in raw sewage to concentrations where they are no longer of concern. *In vitro*
446 bioassays and *in situ* monitoring suggest the occurrence of sources of estrogenic EDCs at WWTP2. *In*
447 *vitro* bioassays and chemical analysis indicate that the secondary treated wastewater from AWRP
448 and WWTP3 would dilution prior to environmental discharge, otherwise estrogenic effects may
449 arise. Note however that RO treatment was very effective at removing the residual estrogenicity and
450 that the RO permeate did not elicit any response in the E-SCREEN assay (<0.02 ng/L EEQ).

451 **4. Conclusions**

452 There was significant cytotoxicity, phytotoxicity and estrogenicity in raw sewage, but most of this
453 activity was greatly reduced during standard wastewater treatment. No biological response was
454 detected to RO water, suggesting that reverse osmosis was a significant barrier to biologically active
455 compounds. Chemical analysis and *in situ* monitoring provided results consistent with the *in vitro*
456 results: chemical analysis confirmed the removal trends observed by the bioanalytical tools, and *in*

457 *situ* sampling did not reveal any evidence of endocrine disruption specifically due to discharge of
458 treated wastewater (although other sources may be present).

459 This study has demonstrated the utility of combining multiple lines of evidence, including chemical
460 analysis, an *in vitro* ecotoxicity toolbox, and *in situ* monitoring in the assessment of water quality.
461 Biomarkers of exposure (*in vitro*) and effect (*in vivo* or *in situ*) are complementary and together
462 provide information with a high level of ecological relevance. It is important however that the assays
463 are used with careful consideration of confounding factors and with a high degree of knowledge of
464 study species and environmental conditions.

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561

562

Tables and Figures

Table 1. Concentrations of androgens, estrogens and herbicides monitored (in ng/L). Values are mean ± SD of 2 independent samples; except where indicated as (a) where n=1. "AWRP" = Advanced Water Reclamation Plant; "WWTP" = Wastewater Treatment Plant; "Inf" = Influent (raw sewage); "1º" = Primary treated wastewater; "2º" = Secondary treated wastewater; "RO" = Reverse osmosis permeate; "IP" = In-plant sample; "Eff" = Effluent (treated wastewater); "NA" = Not available.

		AWRP				WWTP1			WWTP2			WWTP3		
Chemical	**Sampling date**	**Inf**	**1º**	**2º**	**RO**	**Inf**	**IP**	**Eff**	**Inf**	**IP**	**Eff**	**Inf**	**IP**	**Eff**
Androstenedione	Mar-08	340 ± 91	170 ± 2.8	6.3 ± 0.6	<1	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	490 ± 90	400 ± 25	7.2 ± 0.9	<1	54 ± 7.8	14 ± 4.9	17 ± 0.7	180 ± 4.9	14 ± 1.4	10 ± 0.7	270 ± 9.9	15 ± 0.7	7.6 ± 0.4
	Sep-08	310 ± 120	290 ± 7.1	13 ± 0.7	<1	69 ± 7.1	4.8 ± 0.3	3.3 ± 0.7	14 ± 2.1	<1	<1	30 ± 1.4	20 ± 2.1	5.1 ± 0.6
	Mar-09	NA	NA	NA	NA	<1	<1	<1	<1	<1	<1	170 ± 1.4	15 ± 0.1	3.2 ± 0.2
Androsterone	Mar-08	NA	NA	<50	<50	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	NA	NA	<50	<50	430 ± 110	<50	<50	800 ± 74	70 ± 21	67 ± 3.5	5400 ± 1300	86(a)	<50
	Sep-08	1600 ± 690	1500 ± 49	<50	<50	610 ± 17	<50	<50	78 ± 2.8	<50	<50	460 ± 7.7	<50	<50
	Mar-09	NA	NA	NA	NA	<50	<50	<50	280 ± 1.4	<50	<50	1600 ± 14	<50	<50
Etiocholanolone	Mar-08	NA	NA	<100	<100	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	NA	NA	<100	<100	420 ± 32	<100	<100	980 ± 14	180 ± 20	180 ± 52	4900 ± 1500	<100	<100
	Sep-08	3000 ± 270	1600 ± 70	180 ± 25	<100	940 ± 28	<100	<100	150 ± 4.9	<100	<100	1100 ± 17	470 ± 26	180 ± 96
	Mar-09	NA	NA	NA	NA	290 ± 0.7	<100	<100	1000 ± 2.1	<100	<100	2600 ± 7.1	<100	<100
Testosterone	Mar-08	76 ± 2.8	49 ± 1.4	<1	<1	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	110 ± 1.4	130 ± 0.3	<1	<1	5.4 ± 0.5	<1	<1	9.2 ± 0.1	<1	<1	91 ± 1.4	<1	<1
	Sep-08	91 ± 24	100 ± 0.7	<1	<1	7.8 ± 0.6	<1	<1	<1	<1	<1	13 ± 0.7	4.9 ± 0.8	1.2 ± 0.1
	Mar-09	NA	NA	NA	NA	<1	<1	<1	<1	<1	<1	59 ± 0.5	2.5 ± 0.4	2.4 ± 0.1
Dihydrotestosterone	Mar-08	<50	<50	<50	<50	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
	Sep-08	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
	Mar-09	NA	NA	NA	NA	<50	<50	<50	<50	<50	<50	<50	<50	<50
Testosterone propionate	Mar-08	<5	<5	<5	<5	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	Sep-08	17 ± 3.5	22 ± 2.1	<5	<5	9.2 ± 0.8	<5	<5	<5	<5	<5	14 ± 2.8	<5	<5
	Mar-09	NA	NA	NA	NA	<5	<5	<5	<5	<5	<5	<5	<5	<5
17β-Trenbolone	Mar-08	<5	<5	<5	<5	NA	NA	NA	NA	NA	NA	NA	NA	NA

	Jun-08	<5	<5	<5	<5	<5	<5	<5	24 ± 2.1	<5	<5	64 ± 12	<5	<5
	Sep-08	120 ± 27	110 ± 5.7	<5	<5	<5	<5	<5	13 ± 0.7	<5	<5	48 ± 5.6	<5	<5
	Mar-09	NA	NA	NA	NA	<5	<5	<5	<5	<5	<5	<5	<5	<5
17β-Estradiol	Mar-08	<5	<5	<5	<5	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	29 ± 1.4	8[a]	<5
	Sep-08	24 ± 7.8	19 ± 1.4	<5	<5	<5	<5	<5	<5	<5	<5	80 ± 4.9	23 ± 2.8	<5
	Mar-09	NA	NA	NA	NA	<5	<5	<5	<5	<5	<5	<5	<5	<5
17α-Estradiol	Mar-08	<5	<5	<5	<5	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	Sep-08	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	Mar-09	NA	NA	NA	NA	<5	<5	<5	<5	<5	<5	<5	<5	<5
Estrone	Mar-08	NA	220 ± 9.2	25 ± 5.6	<5	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	NA	NA	34 ± 4.2	<5	32 ± 4.9	30 ± 14	37 ± 9.2	430 ± 160	160 ± 26	77 ± 34	NA	95[a]	100 ± 110
	Sep-08	390 ± 180	280 ± 59	67 ± 2.8	<5	74 ± 6.4	18 ± 2.1	13 ± 7.1	31 ± 3.5	20 ± 0.7	<5	NA	60 ± 2.1	<5
	Mar-09	NA	NA	NA	NA	35 ± 2.1	<5	<5	57 ± 1.9	<5	<5	310 ± 5.7	18 ± 0.9	16 ± 0.6
Estriol	Mar-08	<50	<50	<50	<50	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
	Sep-08	820 ± 460	800 ± 7.1	69 ± 5	<50	260 ± 18	190 ± 17	170 ± 20	93 ± 24	140 ± 7.1	58 ± 11	620 ± 17	89 ± 9.2	<50
	Mar-09	NA	NA	NA	NA	<50	<50	<50	<50	<50	<50	<50	<50	<50
17α-Ethinylestradiol	Mar-08	<5	<5	<5	<5	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	Sep-08	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
	Mar-09	NA	NA	NA	NA	<5	<5	<5	<5	<5	<5	<5	<5	<5
Diuron	Mar-08	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	NA	NA	NA	NA	<25	26 ± 0.1	<25	<25	<25	<25	<25	26 ± 18	29 ± 4.1
	Sep-08	38 ± 6.1	44 ± 7.6	50 ± 2.1	<25	<25	<25	<25	<25	<25	<25	<25	32 ± 27	55 ± 8.9
	Dec-08	100 ± 18	150 ± 16	130 ± 5.3	<25	<25	NA	<25	<25	NA	<25	32 ± 2.4	78 ± 11	64 ± 1.9
	Mar-09	NA	NA	NA	NA	39 ± 0.1	36 ± 3.5	<25	30 ± 8	32 ± 0.3	<25	<25	25 ± 17	28 ± 2.5
Metolachlor	Mar-08	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Jun-08	NA	NA	NA	NA	<25	<25	<25	<25	<25	<25	<25	<25	<25
	Sep-08	120 ± 24	76 ± 0.1	26 ± 0.5	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
	Dec-08	32 ± 2.4	29 ± 1.1	<25	<25	<25	NA	<25	<25	NA	<25	<25	<25	<25
	Mar-09	NA	NA	NA	NA	<25	<25	<25	<25	<25	<25	<25	<25	<25

568

569 Table 2. Population and morphometric endpoints for mosquitofish captured upstream and

570 downstream of WWTPs 1 and 2 and at a reference site ("Ref"). "GL" = gonopodial length; "PAL" =

571 pre-anal length (distance from the gonopodium to the snout). Values for GL and PAL are average ±

572 SEM. * Statistically different from corresponding upstream site (α=0.05). † Statistically different from

573 reference site (α=0.05).

		WWTP1		WWTP2		Reference
		Upstream (UP1)	Downstream (DN1)	Upstream (UP2)	Downstream (DN2)	
Males (%)	Sep 2008	28 (14%)	14 (16%)	84 (23%)	11 (12%)	5 (6%)
	Mar 2009	48 (34%)	16 (16%)	40 (31%)	37 (29%)	47 (34%)
	Jul 2009	6 (38%)	5 (22%)	50 (23%)	14 (19%)	9 (21%)
GL (mm)	Sep 2008	6.83 ± 0.11	6.76 ± 0.08	6.79 ± 0.06	7.05 ± 0.09	6.97 ± 0.24
	Mar 2009	6.90 ± 0.07	6.91 ± 0.12	7.04 ± 0.08	7.46 ± 0.07 †	7.15 ± 0.08
	Jul 2009	7.00 ± 0.23	6.75 ± 0.28	6.55 ± 0.68	6.76 ± 0.15	6.78 ± 0.15
PAL (mm)	Sep 2008	8.93 ± 0.17	8.64 ± 0.09	8.67 ± 0.09	9.71 ± 0.22	9.37 ± 0.33
	Mar 2009	9.48 ± 0.10 †	9.04 ± 0.18 *	9.56 ± 0.10	10.13 ± 0.11 †	9.54 ± 0.11
	Jul 2009	9.24 ± 0.29	8.80 ± 0.40	9.11 ± 0.11	9.64 ± 0.18	9.20 ± 0.24

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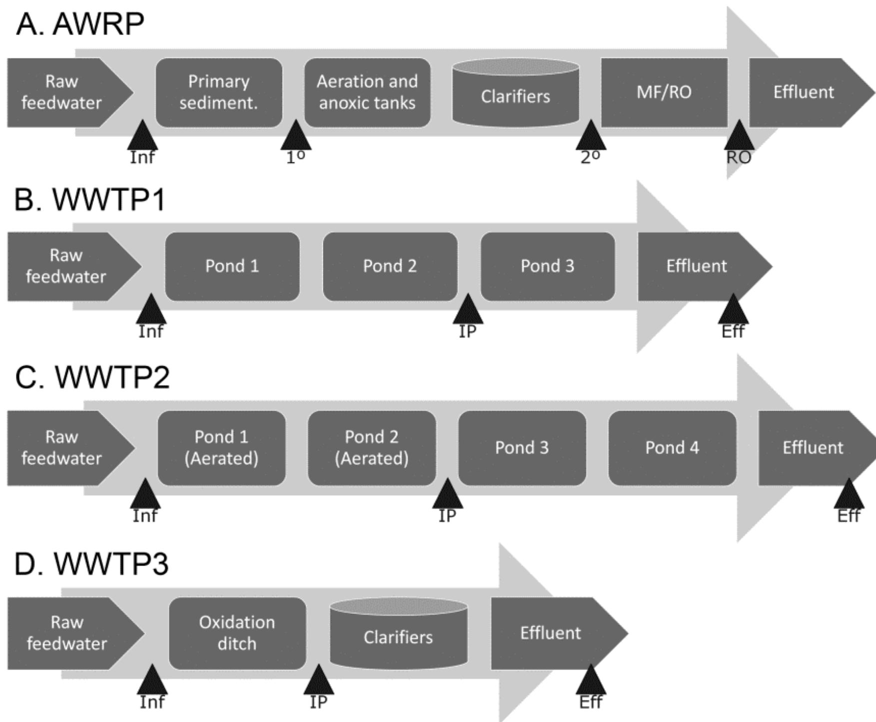

576

577 Figure 1. Treatment train and sampling locations. "AWRP" = Advanced Water Reclamation Plant;
578 "WWTP" = Wastewater Treatment Plant; "Inf" = Influent (raw sewage); "1º" = Primary treated
579 wastewater; "2º" = Secondary treated wastewater; "RO" = Reverse osmosis permeate; "IP" = In-plant
580 sample; "Eff" = Effluent (treated wastewater).

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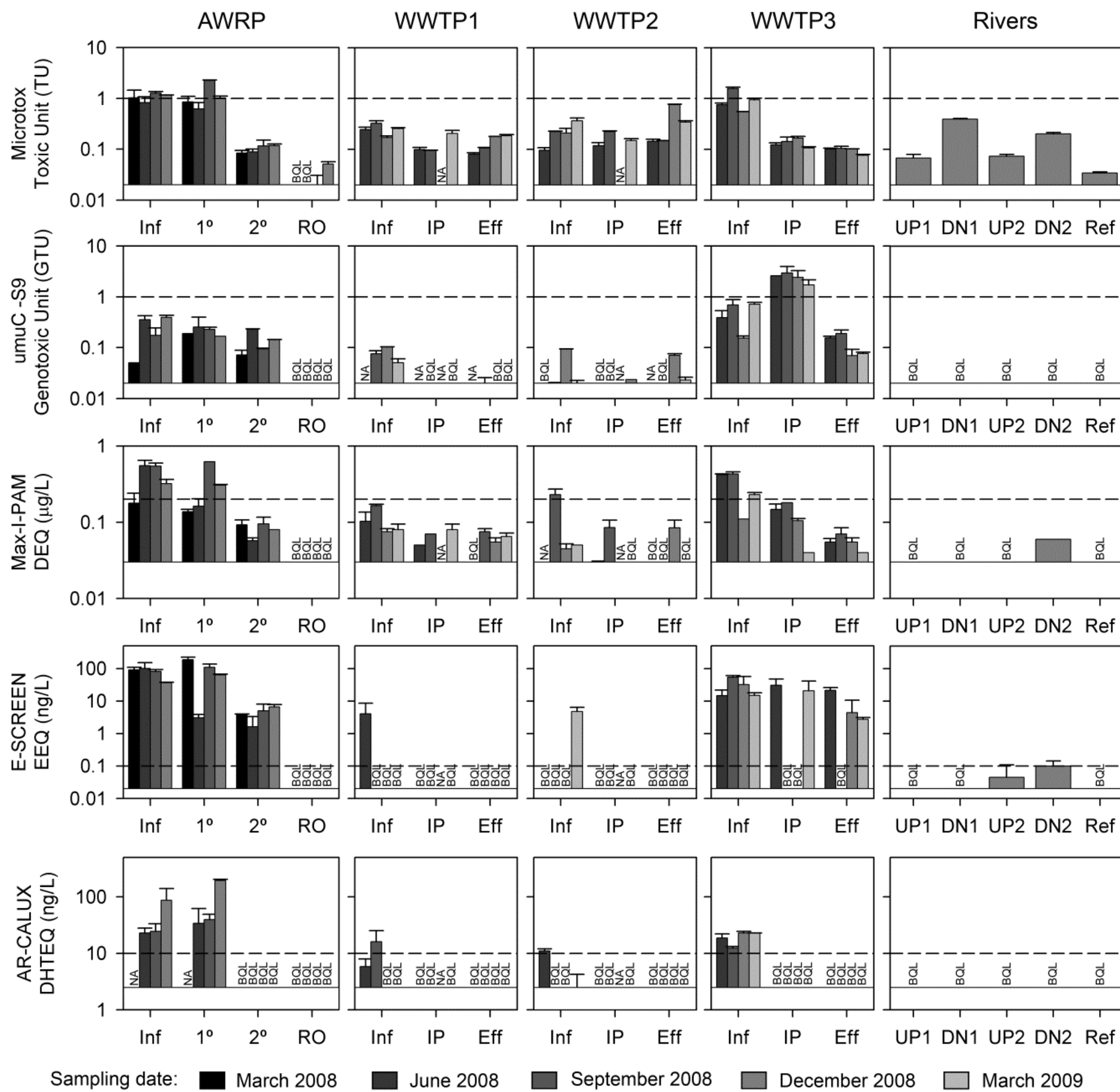

582

583 Figure 2. Bioanalytical results. The dotted line represents the bioassay threshold above which the *in*

584 *vitro* response is expected to lead to adverse effects in exposed organisms. "AWRP" = Advanced

585 Water Reclamation Plant; "WWTP" = Wastewater Treatment Plant; "Inf" = Influent (raw sewage);

586 "1º" = Primary treated wastewater; "2º" = Secondary treated wastewater; "RO" = Reverse osmosis

587 permeate; "IP" = In-plant sample; "Eff" = Effluent (treated wastewater); "UP1" and "DN1" =

588 Upstream and downstream of WWTP1, respectively; "UP2" and "DN2" = Upstream and downstream

589 of WWTP2, respectively; "Ref" = Reference site; "NA" = Not available; "BQL" = Below quantification

590 limit (quantification limit specified in Table 1). n =2 independent samples.

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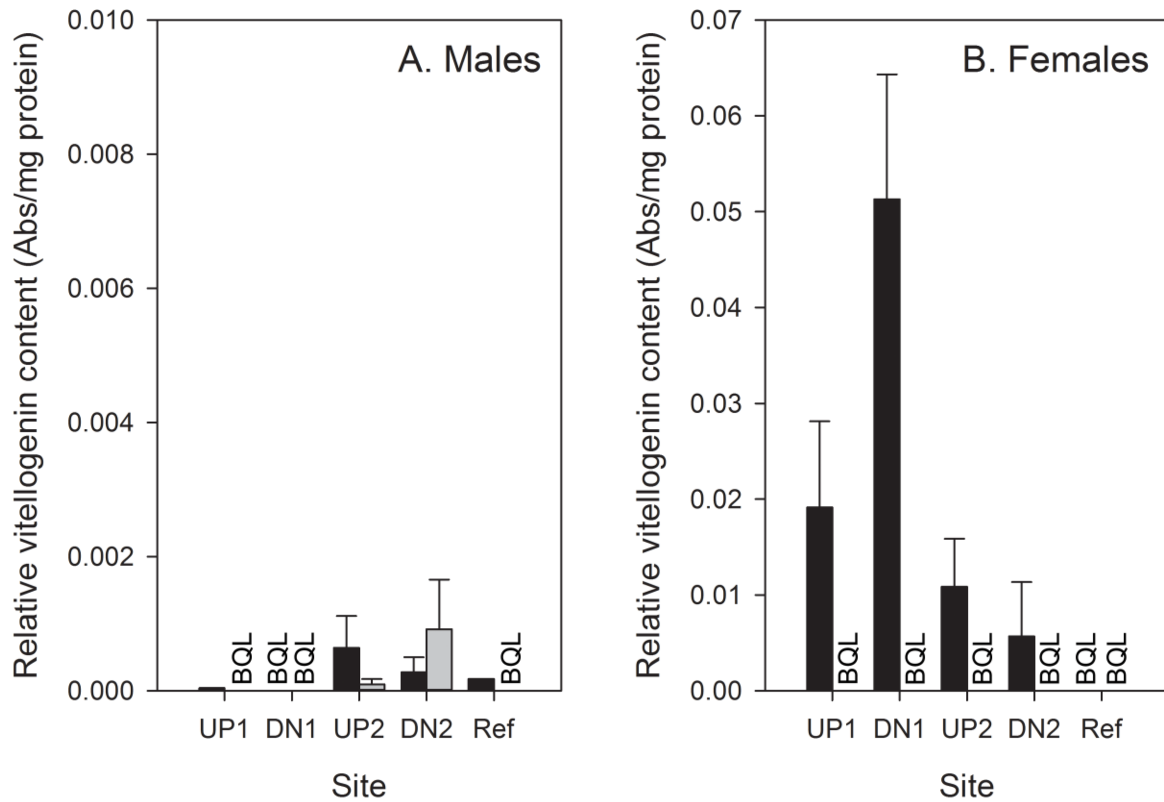


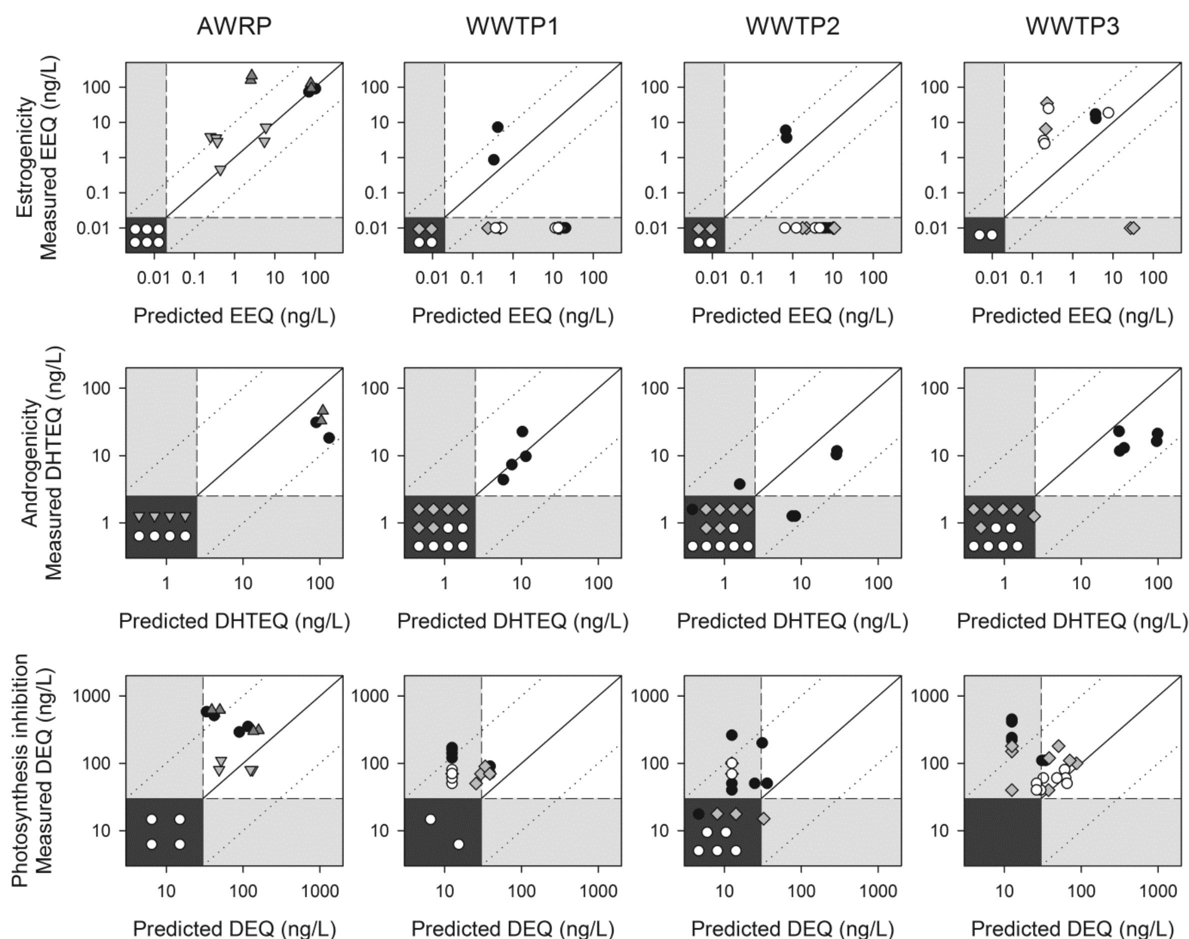
593 Figure 3. Relative vitellogenin content of male (left) and female (right) mosquitofish from upstream

594 and downstream of WWTP1 ("UP1" and "DN1", respectively) and WWTP2 ("UP2" and "DN2",

595 respectively) and at the reference site ("Ref"). Black bars = Summer (Mar 09), grey bars = winter (Jul

596 09). "BQL" = Below Detection Limit.

597



598

599 Figure 4. Comparison of predicted *vs.* measured estrogenicity (as 17β-estradiol equivalent, EEQ; top),
600 androgenicity (as dihydrotestosterone equivalent, DHTEQ; middle) and photosynthesis inhibition (as
601 diuron equivalent, DEQ; bottom). The predicted *in vitro* response was calculated from chemical
602 concentrations of selected compounds multiplied by the potency of each compound in the relevant
603 assay. Predicted estrogenicity was calculated from 17β-estradiol (RP = 1), estrone (RP = 0.012) and
604 estriol (RP = 0.071) concentrations; predicted androgenicity was calculated from androstenedione
605 (RP = 0.057), androsterone (RP = 0.006), testosterone (RP = 0.21), testosterone propionate (RP =
606 0.20) and 17β-trenbolone (RP = 0.50); photosynthesis inhibition was calculated from diuron alone
607 (RP = 1). "AWRP" = Advanced Water Reclamation Plant; "WWTP" = Wastewater Treatment Plant. ● =
608 Influent (raw sewage; "Inf"); ▲ = Primary treated wastewater ("1º"); ▽ = Secondary treated
609 wastewater ("2º"); ○ = Effluent ("RO" and "Eff"); ◇ = In-plant sample ("IP"). Dark gray areas at the
610 bottom left of each graph indicates below detection limit of both methods; light gray area at the

611 bottom and left of each graph indicates areas below the bioanalytical quantification limit and

612 chemical quantification limit, respectively.