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RESEARCH PAPER

CHARACTERISATION OF THE EFFICACY OF AN ADVANCED TERTIARY SEWAGE TREATMENT PLANT TO REMOVE ESTROGENIC COMPOUNDS USING ON-SITE FISH EXPOSURE

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ABSTRACT

The fate and potential risks of endocrine disrupting chemicals (EDCs) in treated sewage effluents remain to be fully characterised. This study applied a combination of chemical analysis and *in vitro* and *in vivo* bioassays to assess the efficacy of an advanced tertiary sewage treatment plant to remove EDCs. Samples from all treatment stages contained measurable concentrations of alkylphenol compounds and bisphenol A (BPA); only the post-sandfilter effluent contained measurable concentrations of the estrogenic steroid estrone (E_1). Bioassay results indicated that the treatment technology of this plant removed estrogenicity to concentrations below the detection limits. Mosquitofish (*Gambusia holbrooki*) and the Australian native rainbowfish (*Melanotaenia fluviatilis*) were exposed to the sandfilter and final effluents and 17β-estradiol as a positive control for seven days on-site at the treatment plant. Vitellogenin mRNA, a biomarker of estrogenicity in fish, was measured by rtPCR. Estrogenicity was detected in the sandfilter effluent by the yeast assay, but the activity was not at a level that modulated up-regulation of vitellogenin mRNA in either exposed fish species.

Keywords: *In situ*; two-hybrid yeast reporter gene assays; mosquitofish; rainbowfish; vitellogenin; rtPCR

INTRODUCTION

Sewage effluents can contain numerous biologically active chemicals including naturally produced, synthetic medicinal, and industrial estrogenic endocrine disrupting chemicals (EDCs) (Auriol et al. 2006; Jalova et al. 2013; Khanal et al. 2006; Liu et al. 2009). The effects of estrogenic EDCs on the reproductive success of fish include reduced egg-laying capacity (Oshima et al. 2003; Santos et al. 2007), decreased hatching success (Shioda and Wakabayashi 2000) and skewed sex ratios

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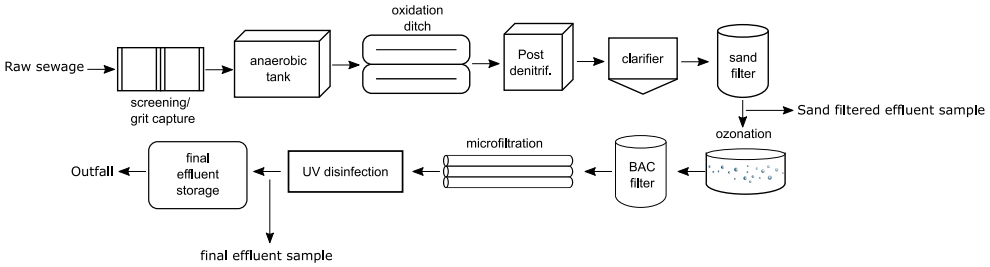

Figure 1. Schematic of the GGSTP and locations of effluent samples for the fish exposure experiments.

of offspring (Nimrod and Benson 1998). Evidence of estrogenic endocrine disruption has been reported in wild populations of fish living in waters receiving treated sewage effluent (Batty and Lim 1999; Jobling et al. 2002; Rawson et al. 2008). However, other studies have found no evidence of endocrine disruption in fish exposed to sewage effluent or in *in vitro* assays (Braga et al. 2005; Carballo et al. 2005; Douxfils et al. 2007; Leusch et al. 2006; Neale et al. 2020).

The application of *in vitro* estrogenic bioassays has demonstrated that advanced tertiary treatment technologies can significantly reduce the concentration of EDCs in sewage effluent (Hamilton et al. 2021; Leusch et al. 2005a; Liu et al. 2009). Advanced sewage treatment technologies are designed to significantly reduce concentrations of recalcitrant micro-contaminants and their potential risk (Boake 2006; Hamilton et al. 2021; Hamilton et al. 2016; Roberts et al. 2015), and provide a valuable option to address increasing water scarcity through the recycling of treated wastewater. However, the integration of treated wastewater back into the water cycle is reliant upon sound data on the concentration and effects of residual EDCs and other micro-contaminants, and robust assessments of the risks they pose to wildlife and humans.

The application of *in vivo* approaches to assess the effects of micro-contaminants upon organisms exposed to treated wastewater is becoming more common (Barber et al. 2007; Garcia-Reyero et al. 2011; Roberts et al. 2015; Vajda et al. 2015). Incorporation of such approaches in bioassessment studies provides information of greater ecological significance than *in vitro* assessment alone (Adams and Tremblay 2003). Vitellogenin (VTG) up-regulation in male fish is a well-established biomarker used in *in vivo* assessment of endocrine disruption in fish exposed to sewage effluent (Aerni et al. 2004; Barber et al. 2007; Jones et al. 2000; Sole et al. 2001; Vajda et al. 2015; Woods and Kumar 2011). When exposed to exogenous (xeno)-estrogens, production of VTG can be stimulated and quantified in the blood plasma or through measurement of hepatic *vtg* mRNA.

The mosquitofish (*Gambusia holbrooki* Girard 1859) was chosen as an *in vivo* test species for exposure to EDCs as it is a small, fast growing, common feral species in Australian waters that has previously been studied for developmental and reproductive changes caused by estradiol exposure (Doyle and Lim 2005; Leusch et al. 2005b). The Murray River rainbowfish (*Melanotaenia fluviatilis* Castelnau 1878) was chosen as a small, fast growing indigenous species that has been used as a native model test species to assess the effects of EDCs (Pollino et al. 2007; Woods and Kumar 2011).

This study assessed the efficacy of advanced tertiary treatment technology to remove estrogenicity from municipal wastewater using combined analytical chemistry and *in vitro* and *in vivo* bioassay approaches. On-site exposure tests were used to determine whether the sandfilter and final effluents would induce *vtg* in males of *G. holbrooki* and *M. fluviatilis* (Vajda et al. 2015).

MATERIALS AND METHODS

Fish flow-through test system

This study was conducted at the Gerringong-Gerroa sewage treatment plant (GGSTP), New South Wales, Australia. The GGSTP is an advanced tertiary treatment plant incorporating continuous backwashing sand filtration, ozonation (O_3), biological activated carbon filtration (BAC), microfiltration and UV disinfection after activated sludge treatment with nitrification/denitrification (Biodenipho™ (Veolia Water Technologies)) and clarification (Figure 1) (Boake 2006).

Two fish exposure experiments were carried out in November 2008 (Expt 1) and February 2009 (Expt 2). Each test was conducted for 7 days.

A series of 20-L glass flow-through treatment tanks were set up on site at the GGSTP. Process feed water from the sandfilter and final effluent outlets and well aerated mains water were plumbed into the test room and distributed to header and treatment tanks.

The treatments consisted of triplicate flow-through exposure tanks containing sandfilter effluent, final effluent (Figure 1), mains water negative controls and a positive control treatment dosed with 17β-estradiol (E_2). Every exposure tank had its own header tank to avoid confounding effects of pseudoreplication and to ensure continuous flow to the exposure tanks, including the periods when the advanced tertiary treatment stages were not running, typically overnight during reduced influent flows. The mains water used in the negative control treatment passed through a partially recirculating system that enabled the header tanks to provide an aerating and aging stage for the removal of any residual chlorine.

The treatment tanks were gravity fed water from their respective header tank to ensure they were continuously replenished. The average flow rate from the header to exposure tanks was 5.4 ± 1.5 L/h during the November 2008 test (Expt 1) and 6.9 ± 1.5 L/hour during the February 2009 test (Expt 2), corresponding to average 99% volume replacement rates of 18.0 ± 4.5 and 13.8 ± 2.7 hours respectively for the November 2008 and February 2009 tests.

The positive control treatments comprised final treated effluent continually dosed with a solution of estradiol in methanol to achieve a nominal exposure concentration of 100 ng/L E_2.

In the November 2008 experiment, each exposure tank was stocked with eight male mosquito fish, and in the February 2009 test, each exposure tank was stocked with four adult male Murray River rainbowfish. Fish were fed daily with commercially available fish food flakes except for the first and last days of the exposure test.

The water temperature, dissolved oxygen, conductivity and pH of each treatment tank were monitored daily.

An eight-channel peristaltic pump (Gilson) fitted with fluorinated ethylene propylene (FEP) tubing was used to take time-integrated daily 24-h composite samples from one of the replicate exposure tanks for each treatment. The 24-hour composite samples were collected in pre-cleaned 4-L glass amber bottles that were packed in ice to reduce analyte degradation. The collected samples were acidified with concentrated H_2SO_4 to pH 2. They were filtered, split into two 1-L samples and extracted at the on-site laboratory immediately upon collection, one for chemical analysis and the other for *in vitro* bioassay.

Chemical analysis

The filtered 1-L wastewater samples collected were spiked with 25 ng of carbon-13 (^{13}C) labelled

surrogate standard mix (3,4-^{13}C2 for αE_2 and E_2, 20,21-^{13}C2 for ethinyl estradiol (EE_2), E_1-^{13}C for estrone (E_1) and estriol (E_3), BPA-^{13}C12 for BPA, triclosan-^{13}C12 (99%) for triclosan, and 4-n-NP-d4 for the nonylphenols).

All samples (for chemistry and bioassay) were extracted through solvent-rinsed and conditioned 500-mg Oasis HLB cartridges (Waters™). Target analytes were eluted with a mixture of dichloromethane: methanol (95:5) and passed directly through a serially connected Florisil column (IST, 500 mg Isolute) containing a 500-mg layer of sodium sulfate to remove residual water. The sample extracts for chemical analysis were further cleaned up using gel permeation chromatography (GPC). Dichloromethane:methanol (95:5) was used as the mobile phase and the fraction containing the target analytes was collected.

Deuterated internal standards (nonylphenol-d8, triclosan-d3, bisphenol-A-d16, E_2-d4, E_1-d4, EE_2-d4) were added to the extracts for chemical analysis which were derivatised by silylation with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) using the method previously described (Labadie and Budzinski 2005). Analysis was performed using an Agilent 6890N gas chromatograph with split/splitless injector, PAL multipurpose auto-sampler, and Agilent 5975 quadrupole mass selective detector (MSD). Mass spectrometric data were acquired in single ion monitoring (SIM) and the concentrations of internal standard, target and surrogate compounds were determined using Agilent Chemstation quantification software with internal standard quantitation as previously described (Hamilton et al. 2016).

Limits of detection (LODs) for the target analytes were determined for the different effluent matrices using a combination of 3x signal:noise ratio as a minimum detectable peak and a relative ion abundance acceptance criterion of 20%.

Two-hybrid yeast bioassay

Sample solvent extracts destined for two-hybrid yeast bioassay analysis were evaporated under a gentle stream of nitrogen and immediately redissolved in 100 µL of dimethyl sulfoxide. The assay uses a genetically modified yeast two-hybrid system where an estrogen nuclear receptor and the TIF2 coactivator have been inserted into the yeast expression plasmid before being transfected to *Saccharomyces cerevisiae* (Y190 strain) (Hamilton et al. 2021; Nishikawa et al. 1999). This bioassay method uses a 96-well plate set-up and the production of β-galactosidase is quantified as the measure of transcriptional activity (Arulmozhiraja et al. 2005). Modifications included the use of zymolase 100T for the enzymatic digestion agent and conducting the assay in Nunc white 96 well plates (In vitro Technologies, Australia). Samples were separately assayed using two yeast-based systems, one transfected with the human ERα (hER) and the other with the medaka fish ER (medER) estrogen receptors (Hamilton et al. 2021). The final results were reported in estradiol equivalence (EEq) for the ECx10 which represents a chemiluminescent signal ten times higher than that of the blank. The EEq corresponds to the concentration of the natural ligand E_2 that would elicit a similar response as the test sample in the assay.

vitellogenin analysis by quantitative PCR

At the end of the fish exposure period, the fish were removed from the exposure tanks, euthanised and the livers were removed and immediately added to 2.0-mL microcentrifuge tubes containing 200 µL RNA*later*TM (Qiagen).

RNA was extracted using either the QIAGEN® RNeasy® micro or mini total RNA isolation kit (QIAGEN) following the manufacturer's protocol. For mosquitofish, *vtg* was quantified using a multiplex one-step PCR reaction using LUX (Invitrogen) labelled primers (Leusch et al. 2005b). For

the rainbowfish, RNA reverse transcription to cDNA was performed using RT primer mix (oligo-dT and random primers) QuantiTect Reverse Transcription Kit (QIAGEN®) prior to quantification of *vtg u*sing QuantiTect SYBR Green PCR mix (QIAGEN®) (Woods and Kumar 2011). Both methods normalised *vtg* up-regulation to the housekeeping gene *18S* ribosomal RNA. The up-regulation of vtg in the fish from the treatment tanks was evaluated graphically using the relative quantification method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen 2001) incorporating standard error of the mean calculations (Bookout and Mangelsdorf 2003). *vtg* Ct values were converted to ΔCt for each fish by subtracting the 18S rRNA Ct. Relative fold change ΔΔCt for each treatment was calculated by subtracting the average ΔCt of the control tank from the average ΔCt for each treatment. Relative up-regulation was calculated as $2^{-\Delta\Delta Ct}$. For statistical analysis ΔCt values for individual fish were used.

Data analysis

The average concentrations of EDCs and the average responses in the two-hybrid yeast assay measured in the 24-hour composite samples were calculated using Kaplan-Meier empirical cumulative distribution functions (K-M ECDF) generated using the NADA package (Lee 2009) in R software 2.9.1 (R Core Team 2009). The difference in responses of the two-hybrid yeast bioassays between the treatment tanks was tested by comparing the K-M ECDFs using the cendiff function in the NADA package (Lee and Helsel 2007). Bonferroni corrected p-values were used for post-hoc paired comparisons.

There was a significant difference ($p<0.05$) between the final effluent replicate tanks in the November 2008 experiment. There was no significant difference in the ΔCt *vtg* up-regulation between fish in the February 2009 replicate tanks (one-way ANOVA or Kruskal-Wallis non-parametric rank test ($p>0.05$)). However, considering the other three treatments were not significant, it was decided to still use the individual fish responses as individual replicates. One-way ANOVA or Kruskal-Wallis tests were also used to test for differences between treatments (control, sandfilter, final (UV) and positive control tanks) for each experiment. Dunnett's test was used for comparing the ΔCt values for each treatment to those of the control tank using the multcomp function (Hothorn et al. 2008) in R and using the sandwich function which uses treatment variance when the variance was significantly different between treatments.

RESULTS

Experimental conditions

In each fish exposure experiment, the temperature was generally similar between the tanks and there were minimal differences in the pH (Table 1). The dissolved oxygen (DO) content was similar between all treatments because aerators were used in all tanks. During exposure experiments, aerators were used in the header tanks of the mains water control treatments to facilitate de-chlorination. This produced variation in the measured DO content between the treatment tanks, and the DO content of the mains water negative control tanks were slightly higher than that in the other tanks (Table 1). The conductivity in the water of the mains control tanks was much lower than that of the treatment tanks, reflecting the higher ionic strength of the effluent feeds compared to the mains treated drinking water (Table 1).

Concentrations of EDCs in the 24-hour composite exposure tank samples

The validation of the analytical chemistry method used to measure selected EDCs is summarised in Table 2. The concentrations of target EDCs measured in the water of the sandfilter and final effluents exposure tanks were low in both fish exposure experiments (Figure 2). E_1 was the only steroid estrogen detected in the sandfilter water samples in both experiments, with an average concentration

Table 1: Water quality conditions measured (Average ± standard deviation) during on-site flow-through fish exposure experiments. Averages are from daily measurements of all tanks during the experimental period.

	pH	Dissolved O_2 (%)	Conductivity (µS/cm)	Temperature (°C)
November 2008				
Control	7.3 ± 0.2	94.6 ± 3.4	161.5 ± 2.3	21.2 ± 0.7
Sandfilter	7.2 ± 0.1	80.7 ± 6.5	594.4 ± 10.6	21.7 ± 0.8
Final Effluent	7.2 ± 0.1	76.8 ± 5.5	590.3 ± 7.5	22.7 ± 0.5
Positive Control	7.2 ± 0.1	81.4 ± 7.2	587.6 ± 21.7	22.5 ± 0.6
February 2009				
Control	7.1 ± 0.1	83.7 ± 2.6	168.1 ± 3.4	24.4 ± 0.6
Sandfilter	7.1 ± 0.2	79.2 ± 6.0	638.4 ± 7.6	24.3 ± 0.8
Final Effluent	6.9 ± 0.1	71.9 ± 4.2	638.5 ± 12.7	25.6 ± 0.7
Positive Control	6.9 ± 0.1	69.9 ± 5.2	638.3 ± 5.4	25.4 ± 0.7

of 7.0 ± 0.8 ng/L. E_3 was detected in one sandfilter effluent sample at a concentration of 40.6 ng/L.

In comparison to the estrogenic steroids, synthetic industrial phenols were regularly detected in the sandfilter and final effluent treatment tank samples in both exposure experiments, but only tertiary NP and BPA were present at concentrations above 10 ng/L. The average concentrations of tertiary NP and BPA were lower in the November 2008 (mosquitofish) than the February 2009 (rainbowfish) experiments. High concentrations of BPA were measured in the mains water control tank treatments for both experiments, being 2237 ± 227 ng/L in November 2008 and reducing to 182.6 ± 46.7 ng/L in February 2009. Analysis of the mains water taken from the on-site laboratory demonstrated it

Table 2: Average ± standard deviation of recovery (%) for different sample matrices and different isotopic labelled EDC analogues. The 13C labelled compounds were used as surrogate recovery standards in the November 2008 and February 2009 fish exposure experiments. The blank sample is to provide a background baseline.

Matrix	triclosan-^{13}C	BPA-^{13}C	E_1-^{13}C	E_2-^{13}C	EE_2-^{13}C
November 2008					
Control	87 ± 9	81 ± 10	79 ± 9	79 ± 9	64 ± 6
Sandfilter	95 ± 22	79 ± 22	92 ± 21	88 ± 24	78 ± 22
Final Effluent	76 ± 10	70 ± 10	73 ± 14	71 ± 11	61 ± 5
Positive Control	80 ± 3	74 ± 3	82 ± 5	107 ± 13	60 ± 4
Blank	80 ± 3	77 ± 5	77 ± 4	78 ± 4	65 ± 3
Mains	90 ± 8	67 ± 10	62 ± 14	58 ± 18	47 ± 11
February 2009					
Matrix		BPA-d16	E_1-d4	E_2-d4	EE_2-d4
Control	NA	104 ± 8	129 ± 41	151 ± 8	180 ± 13
Sandfilter	NA	108 ± 25	125 ± 28	148 ± 28	171 ± 46
Final Effluent	NA	114 ± 5	129 ± 34	160 ± 6	186 ± 22

NA - not assessed.

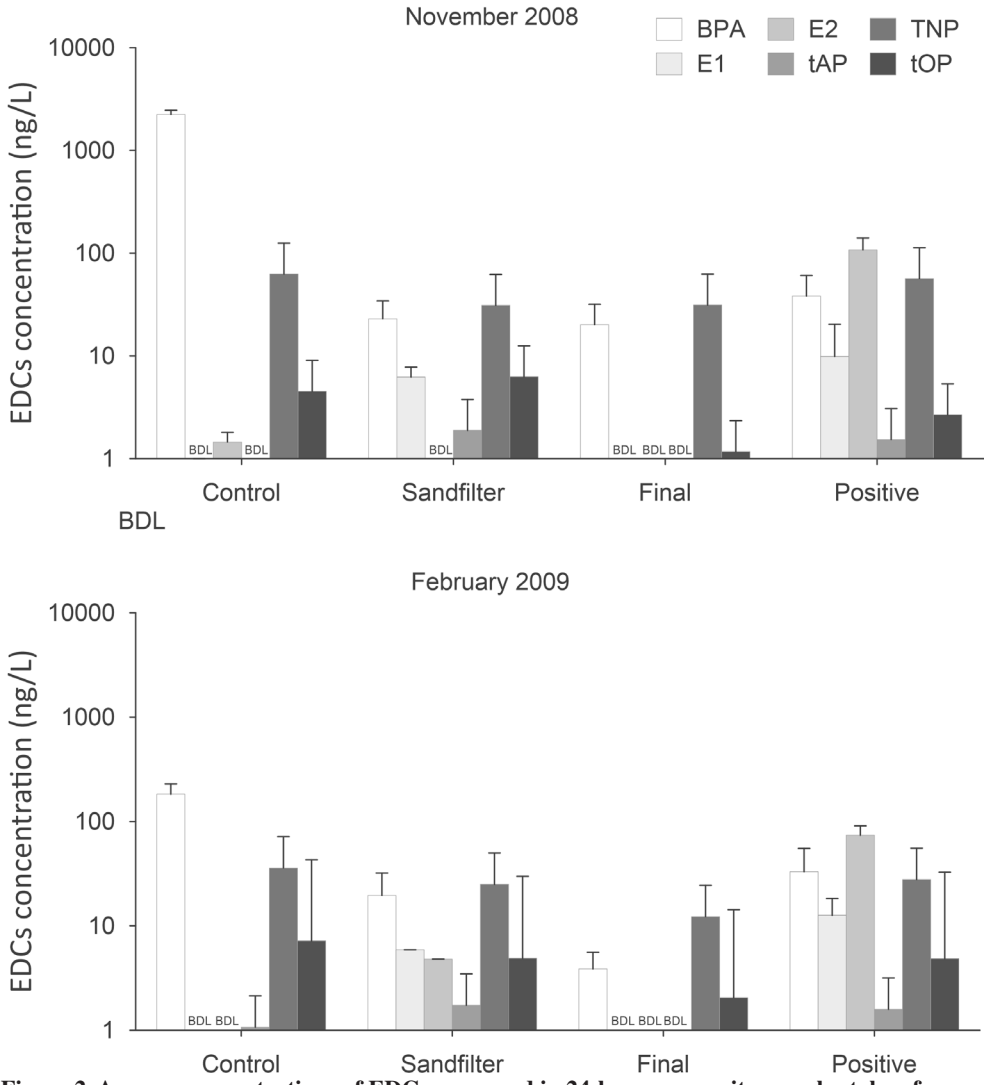

Figure 2. Average concentrations of EDCs measured in 24-hour composite samples taken from a representative tank for each treatment of the fish flow-through exposure experiments in November 2008 and February 2009. Error bars represent the standard deviation. BDL – below detection level. BPA: bisphenol A, E_2: 17β estradiol, TNP: tert-nonylphenol, E_1: estrone, tAP: tert-amylphenol, tOP: tert-octylphenol.

was not the source of BPA. The suspected source of BPA in the mains water treatment tanks is leaching from piping, fittings, and sealants employed in the installation of piping to deliver the process effluents and mains water to the microfiltration room where the exposure tanks were housed.

The concentration of E_2 measured in the mains water positive control treatments was 107.6 ± 32.9 and 73.8 ± 16.9 ng/L, respectively for experiments 1 and 2. The concentration of E_2 measured in the positive control treatment tanks was initially higher and decreased over the 7-day duration of the fish exposure experiments. Conversely, the concentration of E_1 in the mains water positive

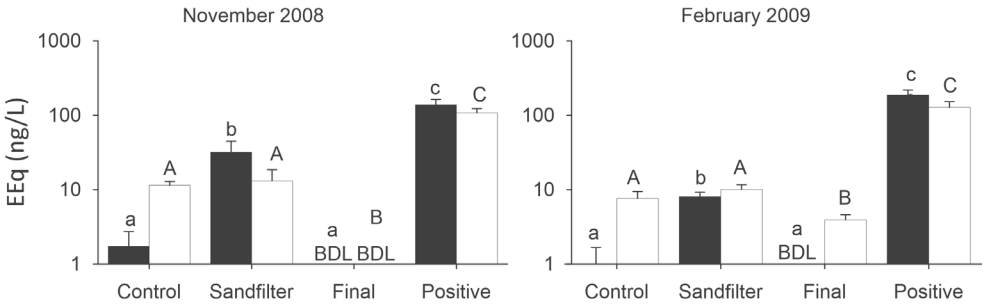

Figure 3. Average estrogenic response in the hER (black) and medER (white) two-hybrid yeast assay for the 24-hour composite samples taken in the November 2008 and the February 2009 fish flow-through exposure experiments. Error bars represent the standard deviation. Columns with the same letter above them are not significantly different (p>0.05). Lower case letters are for hER and upper case for medER responses. BDL – below detection level.

control treatments increased over the course of each exposure experiment, demonstrating that E_2 was increasingly degraded to E_1 over the duration of the exposure experiments. The average concentrations of E_1 measured in the positive control treatments over the course of 2 experiments were 9.9 ± 10.7 and 12.6 ± 5.7 ng/L, respectively.

Two-hybrid yeast response in the 24-hour composite exposure tank samples

The responses in the two-hybrid yeast bioassays demonstrated a low concentration of estrogenicity in the water of the control tanks in both fish exposure experiments (Figure 3).

The medER two-hybrid yeast is more responsive to synthetic phenols than the hER two-hybrid yeast. During all three testing periods the medER two-hybrid yeast displayed a higher response than the hER two-hybrid yeast in the water of the control tanks, indicating that this response may be due to the synthetic phenolic chemicals detected by chemical analysis. The average hER yeast responses in the control tanks were 2.0 ± 0.7 and 1.2 ± 0.6 ng/L EEq for the mosquitofish and rainbowfish experiments, respectively. The average medER yeast responses in the water of the control tanks were 11.4 ± 1.4 and 7.6 ± 1.8 ng/L EEq for the mosquitofish and rainbowfish experiments, respectively.

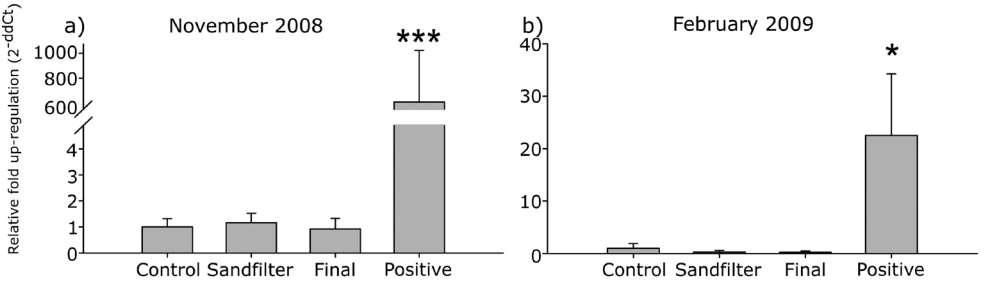

Figure 4: *vitellogenin* upregulation relative to the average *vitellogenin* up-regulation in fish from the control tanks for a) the November 2008 exposure experiment for *G. holbrooki* and b) the February 2009 exposure experiment for *M. fluviatilis*. Error bars represent the standard error of the mean (SEM). * indicates p<0.5 and * indicates p<0.001 for the significant difference to the control by Dunnett test.**

There was a significant difference ($p<0.05$) between treatments in all fish exposure experiments for both the hER and medER two-hybrid yeast responses. In both fish exposure experiments, the sandfilter effluent had a significantly higher hER response than that of the respective control water and final effluent (Bonferroni corrected $p<0.008$). The final effluent also had a significantly lower response in medER yeast than that of the control water and sandfilter effluent ($p<0.008$). Both the medER and hER two-hybrid yeast responses were significantly elevated ($p<0.008$) in the positive control water above that of the control water, sandfilter and final effluents.

vitellogenin up-regulation in males of *Gambusia holbrooki* and *Melanotaenia fluviatilis*

vitellogenin in mosquito fish exposed to either the sandfilter or final effluents was not significantly up-regulated above that of male fish in control water (Kruskal-Wallis rank sum test, chi-squared = 2.0, df = 2, p = 0.352). The relative fold induction was in fact, below one for fish from the sandfilter and final effluent tanks when comparing *vtg* up-regulation to that in the control tank treatments (2-ΔCt) (Figure 4).

vitellogenin up-regulation was observed in fish in the positive control tanks spiked with E_2 in the November (*G. holbrooki*) and February experiment (*M. fluviatilis*) that exhibited significantly lower ΔCt values than the fish in the (negative) control tank (Dunnett multiple contrasts test, mosquitofish $p<0.001$, rainbowfish $p<0.05$).

The fish from the negative control treatments had no detectable *vtg* up-regulation. For statistical analysis, the ΔCt for individual fish from these treatments was calculated as 45 minus 18S Ct. Relative fold induction was calculated using the samples that had detectable *vtg* (n = 2). This was not due to problems with the samples or the qPCR reaction, as the 18S Ct was not significantly different in fish from the different treatment tanks (one-way ANOVA, df = 3,32, F = 0.0192, p = 0.9963) indicating similar reaction efficiencies between treatments. The Ct and delta Ct values for all three fish exposure experiments are presented in Table 3.

Table 3: Average cycle thresholds (Ct) for of *vitellogenin* and the housekeeping gene *18S* and the average normalised *vtg* up-regulation (ΔCt) and change in Ct relative to the normalised *vtg* up-regulation in control tank fish (-ΔΔCt). Error (±) is the standard deviation and brackets contain number of quantifications for treatments that did not have quantifiable *vtg* for all fish.

Treatment	*18S* rRNA Ct	*vtg* mRNA Ct	ΔCt	-ΔΔCt
November 2008 *G. holbrooki*				
Control	8.3 ± 0.5	43.0 ± 0.4 (2)	34.7 ± 0.6	0.0
Sandfilter	8.4 ± 0.6	42.9 ± 1.2 (8)	34.5 ± 1.3	0.21
Final Effluent	8.4 ± 0.4	43.2 ± 1.2 (4)	34.8 ± 1.3	-0.12
Positive Control	8.4 ± 0.8	33.8 ± 2.9 (9)	25.5 ± 3.0	9.2
February 2009 *M. fluviatilis*				
Control	17.2 ± 0.4	26.6 ± 3.6	9.4 ± 3.6	0.0
Sandfilter	16.9 ± 0.3	28.1 ± 3.9	11.2 ± 3.9	-1.8
Final Effluent	17.2 ± 0.8	28.6 ± 4.1	11.6 ± 4.1	-2.1
Positive Control	16.4 ± 0.5	21.4 ± 2.1	5.0 ± 2.1	4.5

DISCUSSION

Although the concentrations of BPA were elevated in the control tank treatments the measured concentration was unlikely to cause up-regulation of *vtg* in the fish in the two fish exposure experiments. Studies on the Japanese medaka (*Oryzias latipes*), the fathead minnow (*Pimephales promelas*) and Chinese loach (*Misgurnus anguillicaudatus*) have shown that BPA is only weakly estrogenic *in vivo* (Kang et al. 2002; Lv et al. 2007; Sohoni et al. 2001). The measured concentration of BPA in the control tank treatments in November (2237 ± 227 ng/L) was 7 to 1400 times lower than BPA concentrations demonstrated to induce *vtg* up-regulation in other fish species.

Based on the potency of BPA as measured in the two-hybrid yeast assay, it would be expected that the concentrations of BPA in the control tank treatments would produce at best a maximum response of 1.0 ng/L EEq. As the medER yeast is more sensitive to synthetic phenols than the hER yeast, its response for the control tank was higher than that for the hER yeast assay. Conversely, in the positive control tank, the higher response in the hER than that in the medER yeast assay was due to the dominance of the steroidal estrogenic compound E_2 driving the high response. The actual responses of both the hER and medER two-hybrid yeasts were higher than what would be predicted from the concentrations of the measured chemicals. As such, it is suspected that the two-hybrid yeast assay may overestimate endocrine disruption (ED) risk as the *vtg* up-regulation did not indicate the same differences between the control and treatment tank samples. This result is in contrast to what has been reported in a review that summarised the findings of wastewater studies correlating *in vitro* and *in vivo* derived data that showed that bioassays underestimated VTG response in whole organisms and caution is warranted with interpretation (Schlenk 2008). Application of partial fractionation of wastewater effluent extracts demonstrated the YES assay response was ten times less than that of plasma VTG induction in Japanese medaka (Huggett et al. 2003). The overestimation of ED risk by the two-hybrid yeast assay may be due to the combined effects of greater sensitivity to synthetic phenols and the clean-up procedure utilised in the current study to reduce the toxicity of the sample extracts to the yeast. The YES assay has also returned positive results for sewage effluents that did not elicit *vtg* up-regulation in male rainbow trout (*Oncorhynchus mykiss*) (Aerni et al. 2004) indicating it may also be a function of differential sensitivities in eliciting *vtg* up-regulation between different species of fish.

Potential overestimation of estrogenic potential derived from the two-hybrid yeast is an important consideration when evaluating the results of the *in vitro* bioassays without the support of chemical analysis or *in vivo* testing. However, the greater sensitivity of the two-hybrid yeast assay to variations in chemical concentrations make it a useful tool when comparing removal efficacy between sewage treatment stages. For example, in this study the two-hybrid yeast bioassays distinguished a difference in ED risk between the sandfilter and final effluents, which was matched by differences in the concentrations of the measured EDCs. The in vivo bioassay was less sensitive than the yeast assays as neither mosquitofish nor rainbowfish males showed significant up-regulation of vtg compared with controls. This is somewhat surprising considering the sandfilter effluent contained traces of E_1 in the mosquitofish exposure experiment. However, while the potency of E_1 for *vtg* up-regulation in male mosquitofish is unknown, in other fish species the potency of E_1 is significantly less than E_2 *in vivo* (Routledge et al. 1998), so it is likely the concentrations of E_1 detected in the sandfilter effluent were too low to up-regulate *vtg* in the exposed mosquito fish.

The estrogenic potencies of various EDCs will vary between species. Alkylphenol ethoxylate (APEO) degradation products, including 4NP and 4tOP, are at least 10,000 times lower in potency than E_2 in the rainbow trout (*O. mykiss)* hepatocytes (Jobling et al. 2006), and the LOECs for NP and OP range between 1-100 µg/L in three different fish species (Mills and Chichester 2005). The fact that E_2 concentrations of 20 ng/L did not significantly up-regulate mRNA *vtg* in male

G. holbrooki (Leusch et al. 2005b) also supports the conclusion that the combined concentration of xeno-estrogens measured in the sandfilter effluent in this study was not sufficient to up-regulate *vtg* in exposed male mosquitofish.

Mosquitofish, including *G. holbrooki* and the closely related *Gambusia affinis*, have been used in several studies assessing estrogenic ED from exposure to treated sewage effluent (Batty and Lim 1999; Leusch et al. 2006; Rawson et al. 2008). *G. affinis* is a sexually dimorphic genus that undergoes gender differentiation within the first few days after birth (Koya et al. 2003). Male mosquitofish develop an elongated anal fin called the gonopodium, which is used to transfer sperm packets to the female gonopore during copulation. Developmental studies using juvenile males have demonstrated that dietary exposure of *G. affinis* to EE_2 (Angus et al. 2005) and waterborne exposure of male *G. holbrooki to* E_2 (Doyle and Lim 2005; Rawson et al. 2008) affects the development of the gonopodium. Exposure to estrogenic compounds has also been shown to decrease sexual activity and impregnation success by adult males, regardless of whether the estrogenic exposure occurred during or after full sexual development (Doyle and Lim 2005; Rawson et al. 2008). The lowest concentration of E_2 where developmental and behavioural abnormalities were detected was 100 ng/L and 20 ng/L respectively (Doyle and Lim 2002; Doyle and Lim 2005). *vtg* mRNA was significantly up-regulated in male G. holbrooki following 8-day exposure to 250 but not 20 ng/L E_2 (Leusch et al. 2005b). This GGSTP study demonstrates that exposure to 107.6 ± 32.9 ng/L E_2 can also produce significant *vtg* up-regulation in male *G. holbrooki.* The actual LOEC for vtg up-regulation in male mosquitofish is therefore likely to be above 20 ng/L but below 108 ng/L E_2.

M. fluviatilis is a native Australian fish species that has been investigated as a potential biomonitor for endocrine disruption. A baseline study (Pollino et al. 2007) found that exposure of *M. fluviatilis to high* E_2 doses (1000 ng/L) reduced the number of eggs laid by females, although the hatchability and size of the larvae appeared not to be affected. Phosphoprotein, a surrogate for VTG, was found to be up-regulated in male fish after 14 days of waterborne exposure at concentrations as low as 30 ng/L E_2. In the testis, γ-glutamyltranspeptidase activity (an indicator of spermatogenesis), was reduced after three days of exposure to 30 ng/L E_2, but after 14 days of exposure, only fish exposed to 1000 ng/L E_2 were significantly affected. In another study, the VTG protein was detected in the liver of male *M. fluviatilis* exposed for 7 days to 500 ng/L of waterbourne E_2 but not 50 ng/L E_2 (Woods et al. 2009). While other markers of estrogenic ED require exposure to concentrations of at least 30 ng/L E_2, hepatic *vtg* mRNA modulation appears to be a very sensitive endpoint, with a LOEC of 10 ng/L and a calculated EC10 of 3.7 ng/L (Woods and Kumar 2011). This was three times more sensitive than ELISA derived protein up-regulation but the two were strongly correlated.

Vitellogenin up-regulation as a biomarker of higher-level biological implications such as developmental and reproductive abnormalities varies greatly between species. Some studies have found *vtg* up-regulation to be more sensitive than physiological, behavioural or reproductive end-points (Kang et al. 2003; Sohoni et al. 2001), while others have found other end-points to be more sensitive than *vtg* up-regulation (Thorpe et al. 2009). In male rainbowfish, *vtg* up-regulation has been shown to be a more sensitive indicator of exposure to EDCs than reproductive measures (Pollino et al. 2007). In male mosquitofish, the situation is less clear, as sexual behaviour is suppressed at 20 ng/L of E_2, but the impregnation rate of females is not significantly reduced until 100 ng/L E_2 (Doyle and Lim 2005). This suggests that *vtg* up-regulation is as sensitive as reproductive output, but not as sensitive as sexual behavioural modification. However, the lack of *vtg* up-regulation in either species when exposed sandfilter or final effluents from the GGSTP does indicate that ED is unlikely to occur in fish exposed to the final treated effluent from GGSTP advanced tertiary treatment plant.

Numerous studies have demonstrated that the release of treated sewage effluent into waterways

is associated with increased *vtg* up-regulation or the intersex (ovotestis) condition in fish studies (Bjerregaard et al. 2006; Diniz et al. 2005; Ma et al. 2005; Porter and Janz 2003; Scott et al. 2006; Thorpe et al. 2009). However, these studies have not considered the EDC removal efficacy of the treatment technologies employed within the STPs being studied.

Although activated sludge treatment is the most common treatment employed in STPs, other treatments and technologies can reduce dramatically the concentrations of different EDCs in final effluents discharged to the environment. For example, the inability of trickling filters to reduce the concentration of EDCs and *vtg* up-regulation in fish exposed to STP effluent at the outfall, and downstream from it, was attributed to the volume and poor quality of the final effluent (Vajda et al. 2008). Many advanced treatment technologies are highly effective at removing EDCs, but few studies have used *in vivo* endpoints to assess the ED risk of effluents subjected to advanced tertiary treatment technologies. Vajda et al. (2015) evaluated endocrine disruption in adult males of the native Australian Murray River rainbowfish *(Melanotaenia fluviatilis)* exposed to effluent from an activated sludge WWTP and water from the Murray River during a 28-d, continuous-flow, on-site experiment. Anti-estrogenicity of effluent samples was detected *in vitro* using YES bioassays (yeast estrogen screen) throughout the experiment, but estrogenicity was limited to the first week of the experiment. Plasma *vtg* concentrations and expression of *vtg* messenger RNA in liver were not significantly affected in fish by 28-day exposure to WWTP effluent. Although no significant *vtg* induction was observed, there was significant suppression of spermatogenesis in fish exposed to wastewater effluent after a 28-d exposure (Vajda et al. 2015). The observed inhibition of gonadal function could be related to the strong anti-estrogenic activity detected by the *in vitro* assay in the WWTP effluent. In the present study, anti-estrogen activity and fish histopathological analyses were not carried out. It was reported that the addition of a sandfilter stage to conventional activated sludge treatment improved general water quality and further reduced the concentrations of E_1 and BPA, but ozonation was required to eliminate *vtg* up-regulation in juvenile rainbow trout (Gunnarsson et al. 2009). Our results demonstrate that the effluent collected after sand filtration did not contain EDCs at concentrations high enough to up-regulate *vtg* in mosquitofish and rainbowfish males.

The continuous backwash sandfilter treatment stage at GGSTP did not provide additional removal of EDCs beyond that achieved by the preceding activated sludge, denitrification and clarification stages (Hamilton et al. 2016). Activated sludge treatment removed the largest proportion of EDCs and reduced their concentrations below those that up-regulate *vtg* in mosquitofish and rainbowfish males. Assessing the efficacy of STPs remains a priority to minimise the risk to receiving environments and the use of biological methods covering multiple mechanisms of toxicity and new approaches like transcriptomics will assist managers optimising treatments (Qin et al. 2021).

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