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**Seasonal variation in oestrogenic potency and biological effects of wastewater treatment works effluents assessed using ERE-GFP transgenic zebrafish embryo-larvae.**

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## **Abstract**

Effluents from wastewater treatment works (WwTW) exhibit both temporal and spatial variation in oestrogenicity, however few studies have attempted to quantify how this variation affects biological responses in fish. Here we used an oestrogen-responsive green fluorescent protein (ERE-GFP) transgenic zebrafish (*Danio rerio*) to quantify oestrogenic activity and health effects for exposure to three different WwTW effluents.

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Endpoints measured included survival/hatching rate, GFP induction (measured in target tissues or *gfp* mRNA induction in whole embryos) and *vtg* mRNA induction in whole embryos. Exposure to one of the study effluents (at 100%), resulted in some mortality, and exposure to all three effluents (at 50% and 100%) caused decreases in hatching rates. Higher levels of *vtg* mRNA corresponded with higher levels of steroidal oestrogens in the different effluents, with lowest-observed-effect concentrations (LOECs) between 31 ng/L and 39 ng/L oestradiol equivalents (EEQs). Tissue patterns of GFP expression for all three WwTWs effluents reflected the known targets for steroidal oestrogens and for some other oestrogenic chemicals likely present in those effluents (i.e. nonylphenol or bisphenolic compounds). GFP induction was similarly responsive to *vtg* mRNA induction (a well-established biomarker for oestrogen exposure). We thus demonstrate the ERE-GFP transgenic zebrafish as an effective model for monitoring the oestrogenic potency and health effects for exposure to complex mixtures of chemicals contained within WwTW effluents.

Cooper et al \_Highlights

- ERE-GFP zebrafish applied to quantify the oestrogenicity of WwTW effluents.
- GFP tissue patterning was reflective of the oestrogenic content of the WwTW effluents.
- Tissue oestrogenic responses varied seasonally and between different WwTW effluents.
- GFP induction was similarly responsive to *vtg* mRNA induction.
- ERE-GFP zebrafish provide an effective biosensor for health effects monitoring of complex mixtures.

## 1. Introduction

Globally, it has been estimated that each year over 450 km<sup>3</sup> of domestic, industrial and agricultural wastewater is released into our aquatic environments, which in turn

requires 6000 km<sup>3</sup> of freshwater for treatment – an amount equivalent to two thirds of the world's usable surface freshwater (Shiklomanov, 2000). Dilution of these wastewaters varies both regionally and seasonally, and in some instances, effluents are released undiluted (and even untreated) into natural freshwaters (Corcoran et al., 2010). As the demand for clean water increases, and because of uncertainties in freshwater supply due to climate change, this will put further pressures on the use of freshwater for the dilution of effluent discharges and thus potential threats of Wastewater Treatment Work (WwTW) effluents to aquatic wildlife are of increasing global concern.

Evidence from both field and laboratory studies have shown a clear link between exposures to WwTW effluents and adverse health effects in aquatic wildlife. In fish, these effects include altered reproductive development (Tetreault et al., 2012; Thorpe et al., 2009), genotoxic damage (Rocco et al., 2012; Wilhelm et al., 2018), alterations in immune function (Borgia et al., 2018; Liney et al., 2006), cytotoxic and oxidative damage (Gagné et al., 2006; Lacaze et al., 2017b), nephrotoxicity (Liney et al., 2006) and alterations in behaviour (Gauthier and Vijayan, 2020; McCallum et al., 2017).

Effluents from WwTWs contain highly complex mixtures of chemicals, comprising of both natural and synthetic organic substances, metals and trace elements, making it inherently difficult to establish specific chemical cause and effect relationships for the health conditions seen in exposed aquatic wildlife. Reported disruptions in reproductive processes in fish exposed to these effluents, that have included altered sex hormone levels (Jeffries et al., 2008; Lacaze et al., 2017a), intersexuality and demasculinisation (Bahamonde et al., 2015a, 2015b; Sanchez et al., 2011), decreased gamete production and reduced fertility (Cavallin et al., 2016; Teta and

Naik, 2017), have been shown to be caused by endocrine disrupting chemicals (EDCs).

Steroidal oestrogens (both natural and synthetic) are some of the most potent EDCs found in WwTW effluents and many studies have confirmed their ability to disrupt sexual development and reproduction in freshwater fish, including for environmentally relevant exposures (Kidd et al., 2007; Schwindt et al., 2014). In humans, it has been suggested that exposure to oestrogenic chemicals can result in reduced sperm count/quality, disruptions in the development of the reproductive tract and oestrogen dependent cancers (Jørgensen et al., 2012; Yuan et al., 2019) as well as effects on the immune system, obesity and cardiovascular disease (Hatch et al., 2010; Lang et al., 2008; Sweeney and Voelkel, 2009).

The oestrogenic activity of WwTW effluents varies temporally and is influenced by the composition of the wastewater entering the works, the type of treatment process employed (Ben et al., 2018; Sun et al., 2017), levels of rainfall, and in temperate climates by seasonal differences in temperature that can affect both the types of microorganism present and their efficacy in biodegrading chemicals (Cruikshank and Gilles, 2007; Phillips et al., 2012). Various *in vitro* assay systems have been adopted for the screening and monitoring of environmental oestrogens in WwTWs including the yeast oestrogen screen (YES) assay (Routledge and Sumpter, 1996) and E-Screen assay (Legler et al., 1999) both of which involve a single oestrogen receptor (ER) subtype (there are two ERs in humans - ER $\alpha$  and ER $\beta$ , and three forms in fish – ESR1, ESR  $\beta$ 1, ESR  $\beta$ 2). These assays provide information on levels of oestrogen activity at the level of a single receptor interaction site but extrapolation to possible health effects is extremely difficult. More recently, several transgenic biosensor fish lines have been developed for testing for oestrogenic activity that are both sensitive to

estrogen exposure and can provide deeper insights into the potential health impacts of oestrogenic compounds and their mixtures (Bakos et al., 2019; Green et al., 2018, 2016; Lee et al., 2012). Transgenic biosensor fish lines can also be used to support the adverse outcome pathway (AOP) framework because they enable direct links to be drawn between binding and activation of a specific receptor or enzyme and a phenotypic outcome (e.g. Brown et al., 2019).

In this study, we applied an oestrogen responsive element (ERE) – green fluorescent protein (GFP) transgenic zebrafish (*Danio rerio*) (Lee et al., 2012), that detects oestrogenic responses for all three ER subtypes, to investigate for responding target tissues and health effects for exposure to three different WwTW effluents at two dilutions (50 and 100%) at time points over the course of a year. Exposures to 10 ng/L EE2 were included in each experiment serving as a positive control. We also compared the sensitivity of the fluorescence responses in the transgenic zebrafish model with vitellogenin (VTG) mRNA induction, a well-established biomarker for oestrogen exposure in fish, for the effluent and EE2 exposures.

## 2. Materials and methods

### 2.1 Test Animals and Husbandry

All fish were maintained under approved protocols, according to the UK Home Office regulations for the use of animals in scientific procedures. Stock ERE-GFP transgenic zebrafish (developed at the University of Exeter; 3xERE:Gal4ff and UAS:GFP; (Lee et al., 2012)) employed in this study were raised under the following conditions: water temperature  $28.5 \pm 1$  °C, with a 12-hr light, 12-hr dark cycle photoperiod regime with gradual dawn and dusk transitions of 30 minutes. Fish were fed live newly hatched Brine Shrimp (*Artemia*) and frozen Gamma Omega 3 Enriched

Brineshrimp (supplied by Zebrafish Management Ltd., Winchester, UK). The supply of water to the laboratory aquaria was subjected to reverse-osmosis with subsequent addition of salts (as described in OECD Guideline 203; (OECD, 2019)). In the experimental exposures (monitored daily) the aquarium water temperature ranged between 26.5 and 27.6 °C, the pH ranged between 6.8 and 7.7, the water conductivity was between 200 and 255  $\mu\text{S}/\text{cm}$  and the dissolved oxygen concentrations were > 80% of the air saturation value.

## *2.2 Study WwTWs effluents and effluent collection*

We studied the effluents from three WWTWs located in England. The total and industrial population equivalent (PE; or unit per capita loading is a means of quantifying the organic strength of wastewater discharges and reflects the net waste of the surrounding human population and industrial facilities, which in the European Union countries is based on a biological oxygen demand (BOD) of the water of 60 g per person/per day). PEs for each of the WWTWs were obtained from the water utility responsible for their operation. The WWTWs studied here are typical of those found in the UK, in that they receive waste primarily from domestic sources, with some industrial/trade waste also. Full details of total population equivalent, types of treatment processes and the types of industrial/trade discharges at each site are provided in Table 1. Dissolved oxygen concentrations for all consignments of effluent were above 80%, and pH values for the effluent consignments ranged between 6.5 and 7.2 for WwTW1, 6.9 and 7.3 for WwTW2 and 7.4 and 7.7 for WwTW3. Conductivity ranged from 590-980  $\mu\text{S}/\text{cm}$  for WwTW1, 4122-7173  $\mu\text{S}/\text{cm}$  for WwTW2 and 460-590  $\mu\text{S}/\text{cm}$  for WwTW3. A range of physiochemical parameters were measured, including temperature, rainfall, dissolved/suspended solids, nitrogen and

phosphorus levels, and biochemical and chemical oxygen demand (BOD/COD) concentrations and are provided in Table 1.

Samples for both analytical chemistry and for embryo-larval exposures comprised of 24-hr WwTW effluent composites (10 L per sampling occasion) collected four times a week from the study sites. Samples were collected using an automatic sampler, in which the sampling frequency was set to every 20 min (i.e. 72 individual grab samples in total/24 hr). All samples were subsequently transferred to 2.5 L glass Winchester bottles, that had been previously acid washed and rinsed with acetone (analytical reagent grade, Fisher Chemicals, Loughborough, UK) and dichloromethane (HPLC grade, Fisher Chemicals), to remove any traces of contaminants, and immediately transported back to the university in cool boxes. Upon arrival at the lab, samples needed for the fish exposure work were slowly acclimated to the desired test temperature of 28 °C, stirred and aerated 1hr prior to exposure, and remaining samples were stored at 4 °C to minimise the risk of oestrogen degradation until required for renewal of the exposure medium.

### *2.3 Analytical measurement of concentrations of oestrogenic chemicals; Estrone (E1), 17 $\beta$ -estradiol (E2), and 17 $\alpha$ -ethinyloestradiol (EE2)*

Analytical chemistry was conducted for all WwTW sample collections. For this, 3 L of effluent samples were stabilised immediately upon collection of the WwTW effluent with methanol (HPLC grade, Fisher Chemicals; 5% final concentration) and acetic acid (HPLC grade, Fisher Chemicals; 1% final concentration) and stored at 4 °C prior to Solid Phase Extraction (SPE). The 24 hr composite samples were divided into three 1 L replicates and spiked with internal standards (ISTDs) containing deuterated estrone- (E1d4), deuterated 17 $\beta$ -estradiol (E2d4) and deuterated 17 $\alpha$ -ethinyloestradiol



(EE2d4). Deuterated E1d4 and E2d4 were obtained from CDN Isotopes (Quebec, Canada), and EE2d4 from Cambridge Isotope Labs (Andover, MA, USA). The samples spiked with ISTD were then pre-filtered through glass wool and filter paper Whatman No1 (Whatman, Maidstone, UK) to remove any large particulates, prior to extraction onto pre-conditioned (with HPLC-grade methanol and water, both Fisher Chemicals) 1 g Oasis HLB cartridges (60 µm particle size; Waters, Manchester, UK). The residue was then cleaned up using DSC-NH<sub>2</sub> cartridges according to Flores et al. (2008). Full details of the SPE method can be found in the Supplementary Information. Subsequent measurement of E1, E2, EE2 concentrations was performed via ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) using a Waters Acquity UHPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). The method detection limits (MDL) for E1, E2 and EE2 were 0.2 ng/L, 0.4 ng/L and 0.5 ng/L, respectively. The method quantification limits (MQL) for E1, E2 and EE2 were 0.6 ng/L, 1.2 ng/L and 1.5 ng/L, respectively. Details of the LC-MS method can be found in the Supplementary Information. All positive controls (EE2) and controls were treated and extracted under the same conditions.

#### *2.4 Estimation of the relative oestrogenic potencies of the WwTW effluents tested*

*(based on the measurements of E1, E2, and EE2)*

A ratio of relative potencies of 1:0.75:40 for E2:E1:EE2 were used to calculate the equivalent concentrations of E2 (EEQs) of the WwTW effluents for use in comparing the total steroidal oestrogenic content with responses for *gfp* and *vtg* induction levels of their respective mRNA in the exposed ERE-GFP zebrafish. These relative oestrogenic potencies were chosen based on information in the literature for the

induction of VTG in cyprinid fish *in vivo*, including zebrafish (Jobling et al., 2006; Van den Belt et al., 2004). This toxic equivalency approach is recommended for assessing the risk of oestrogenic chemical mixtures, such as wastewater effluent, and is based on the assumption that the combined effects of endogenous oestrogens are additive in their effects (Thorpe et al., 2006).

## 2.5 Experimental Exposures

ERE-GFP zebrafish embryos (n = 50 per treatment x 3 replicates in 400 ml sterile glass dishes) were exposed from fertilisation to 96 hours post-fertilisation (hpf) to one of the following treatments: a dilution water control, a reference oestrogen positive control (EE2, at nominal concentration of 10 ng/L) or a WwTW effluent at either 100% or diluted to 50% with embryo culture water (prepared according to the ISO-7346/3 guidelines (ISO, 1996). Each treatment was performed in triplicate. Stock solutions of EE2 ( $\geq 98\%$  purity, purchased from Sigma, Poole, UK) were prepared in acetone (10 mg/L) and stored in a dark cold-room (4 °C) to minimise the risk of degradation. Solvent free working solutions were prepared in embryo culture water the day prior to use, by pipetting the required amount of stock solution into a glass bottle and evaporating the solvent away under a stream of nitrogen. In the embryo cultures, half of the treatment medium was replaced every 24 hr (with freshly collected effluent) and all treatments were gently aerated using a glass pipet connected to tubing fed through each dish lid to ensure that the dissolved oxygen concentration remained greater than 80% of the air saturation value.

## 2.6 Fish sampling and analysis of phenotypic endpoints

All animal use protocols were carried out ethically in accordance with U.K. Home Office guidelines (Animals (Scientific Procedures) Act 1986). Fish were sacrificed by terminal anaesthesia with benzocaine (0.5 g/L; ethyl-p-aminobenzoate; Sigma). Survival rates were assessed for each treatment every 24 hr (see supplementary information Table S1). All surviving embryos in control groups across all experiments hatched by 72 hpf, so this was subsequently used to determine hatching rate (success) in other treatments groups. Subsequently, three pooled sets of embryos were sampled at 96 hpf and examined for GFP expression using Western blot analysis (n = 30 fish per treatment), for *vtg* and *gfp* mRNA expression (n = 45 fish per treatment).

## 2.7 Image analysis

Using fluorescence microscopy (Zeiss Axio Observer.Z1; Zeiss, Cambridge, UK), GFP was measured in exposed zebrafish at 24, 48, 72 and 96 hpf (n = 15 per treatment). GFP was visualised using the same parameters with a 10x objective, employing the Axiovision digital software programme for the production of images. Exposure times employed were kept consistent and were dependent on the tissue studied (due to the varying levels of fluorescence intensity seen in the different target tissues; 50 ms for head region, 20 ms for mid trunk region, and 400 ms for tail section). Prior to live imaging all fish were anaesthetised with 0.4% tricaine, mounted in 4% methylcellulose in a glass-bottom dish (35 mm) and oriented in dorsal, ventral and lateral views. All images were aligned, and the contrast adjusted using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA) keeping the same intensity for all samples.

### *2.8 Quantification of GFP (EGFP) using image analysis*

GFP in the individual tissues was quantified using ImageJ™ software (<http://rsb.info.nih.gov/ij/>). For each image, tissues of interest were masked (outlined) manually to provide a specific quantifiable region of interest (ROI). The mean pixel intensity value from the ROI was then used as a means of quantifying the fluorescence response, and this was applied in a consistent manner across all individuals analysed. Background was subtracted using the ImageJ rolling ball algorithm which removes any spatial variations of the background intensities as described by Sternberg (1983). For fluorescence quantification in the somites, three random measurements were taken employing a plug-in Python macro. GFP expression values are presented as a fold-increase in GFP above that in unexposed controls.

### *2.9 Quantification of GFP (EGFP) using Western blot analysis*

Fish were sampled at 96 hpf to quantify whole body GFP using Western blot analysis, according to the protocol described previously (Lee et al., 2012). Briefly, 10 embryos were dissolved in reduced 2× lithium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA, USA), incubated at 70 °C for 15 mins and then homogenised using a hand homogenizer. Samples were then centrifuged, applied to electrophoresis using NuPAGE NOVEX 4–12% Bis-Tris gel (Invitrogen) and the separated proteins transferred onto a nitrocellulose membrane. This membrane was then incubated with blocking solution (5% skimmed milk in 1× phosphate-buffered saline + 0.1% Tween 20 (PBSTw), washed with ultrapure water (ddH<sub>2</sub>O) and incubated overnight at 4 °C with

primary rabbit anti-GFP antibody (1:2,500) (AMS Biotechnonology, Abingdon, UK). Subsequently, the membrane was washed with 1× PBSTw, incubated with the secondary antibody (horseradish peroxidase–conjugated goat anti-rabbit IgG (1:2,000); Invitrogen) and then washed with 1× PBSTw. For GFP detection, western blotting luminol reagent (Thermo Scientific) was used. The signal strength of the GFP and alpha tubulin bands were evaluated with Image J software (<http://rsbweb.nih.gov/ij/>) and the GFP signal standardised to the intensity of alpha tubulin (used as loading control) and shown as a fold increase in GFP levels in exposed embryos above that in unexposed embryos.

## 2.10 Quantification of the relative transcript levels of *gfp* and *vtg* in whole embryonic larvae using RT-qPCR

### 2.10.1 RNA extraction and cDNA synthesis

Samples for measurement of whole body *vtg* and *gfp* mRNA were immediately snap-frozen in liquid nitrogen upon collection and stored at –80 °C until use. Total RNA was extracted from all samples using Trizol Reagent (Sigma), according to the manufacturer's instructions. The amount of RNA was quantified using a NanoDrop 1000 spectrophotometer and RNA purity determined through the measurement of the A260/A280 and A260/A230 ratios, respectively. Reverse transcription was subsequently carried out by incubating 1 µg RQ1 DNase treated (Promega, Southampton, UK) total RNA with 5 mM random hexamers (Eurofins MWG Operon, Ebersberg, Germany) 10 mM dNTPs and 1 µl MMLV-Reverse transcriptase (Promega) in the appropriate buffer, following the manufacturer's instructions.

### 2.10.2 Real-time quantitative PCR

RT-qPCR was carried out on cDNA samples for each treatment group and sampling point for selected target genes with the iCycler iQ Real-time Detection System (Bio-Rad Laboratories Inc., CA, USA). Gene-specific primer sequences for *vtg* and *gfp* were designed with Beacon Designer 7.2 software (Premier Biosoft International, Palo Alto, USA) and obtained from Eurofins MWG Operon. In brief, primer pairs were optimised for annealing temperature ( $T_a$ ), specificity confirmed by melt curve analysis, and the detection range, linearity and amplification efficiency (E) established using serial dilutions of zebrafish cDNA. The primer sequences, PCR product sizes, annealing temperatures and PCR efficiencies for each primer pair are shown in the supplementary information (Table S2). RT-qPCR was carried out using iTaq Universal SYBR Green Supermix (Bio-Rad), with an initial activation step of 95 °C for 15 min followed by 40 cycles of denaturation (95 °C, 10 s) and annealing (appropriate  $T_a$ , 45 s) and final melt curve analysis. Expression levels of ribosomal protein L8 (*rpl8*) were used as a 'housekeeping' gene to normalise the expression of other genes and determine efficiency-corrected relative expression levels, as described previously (Filby and Tyler, 2005). All samples were analysed in triplicates and a template-minus negative control was run on each plate to verify the absence of DNA contamination.

### 2.11 Statistical analysis

In all cases, data were checked for normality using the D'Agostino-Pearson test and for homogeneity of variance using Bartlett's or Levene's test prior to statistical analysis. Data meeting these criteria were analysed using parametric tests, e.g. analysis of variance (ANOVA) procedures, whilst data that did not conform to

normality were subject to nonparametric tests, e.g. Kruskal–Wallis. Post hoc analysis was performed against controls using the Dunnett's test and with the Tukey test for between-treatment comparisons. Data were transformed, where necessary, using  $\log_{10}$ . Any significant differences from expected sex ratios or stages of gonadal development were tested using the chi-square test. The relationship between levels of *gfp* vs *vtg* expression were measured using Pearson's correlation test. All statistical analyses were run in GraphPad Prism 7 (GraphPad Software, Inc., San Diego, USA). Statistical significance was accepted as  $p < 0.05$  for all comparisons and values are quoted as mean values and ranges as standard error (SE). To calculate monthly mean estrogen concentrations, concentrations that were over the limits of detection ( $\geq$  MDL) but below the limits of quantification ( $<$  MQL) were assigned the MDL value. Concentrations below the MDL were considered to be zero (David et al., 2018).

### 3. Results

#### 3.1 Measured concentrations of major steroidal oestrogens in the test effluents

Analytical measurements for E1, E2 and EE2 (the three principal oestrogens occurring in WwTW effluent) were used to characterise the steroidal oestrogenic content of the different effluents. Measured concentrations of E1 and E2 found in all three WwTW effluents varied across the different collection time-points (Table 1). Except for one occasion, the synthetic oestrogen, EE2 was measured  $<$  MDL (0.5 ng/L) in all effluent samples.

For WwTW1, concentrations of E1 in the four composite samples collected over the course of 4 days ranged between 14.0 and 18.41 ng/L during March 2014 (mean  $16.7 \pm 1.1$  ng/L), lower in October 2014 (between 3.9 and 5.0 ng/L; mean  $4.4 \pm 0.2$  ng/L) and were considerably lower during July 2014 (between  $<$  MDL and 1.3 ng/L;

mean  $0.6 \pm 0.24$  ng/L) and January 2015 (between  $< \text{MDL}$  and  $0.6$  ng/L; mean  $0.6 \pm 0.04$  ng/L). E2 concentrations ranged between  $< \text{MDL}$  and  $2.4$  ng/L (annual mean  $0.16 \pm 0.11$  ng/L) over the entire exposure period with the highest recorded levels seen during July 2014 ( $1.6 \pm 0.42$  ng/L). The level of total oestrogenic activity for this WwTW effluent ranged between  $0.6$  ng/L to  $13.7$  ng/L EEQs.

The WwTW2 effluent was the most strongly oestrogenic of the effluents tested, where concentrations of E1 ranged between  $23.4$  and  $56.9$  ng/L during January 2015 (mean  $36.7 \pm 6.05$  ng/L) and were at their lowest (between  $< \text{MDL}$  and  $2.1$  ng/L) during October 2014 (mean  $1.1 \pm 0.42$  ng/L). E2 concentrations ranged between  $< \text{MDL}$  and  $4.0$  ng/L (annual mean  $2.6 \pm 0.0.15$  ng/L) over the entire exposure period with the highest recorded levels seen during March 2014 ( $4.0 \pm 0.09$  ng/L). Levels of total oestrogenic activity measured at this site ranged between  $1.1$  ng/L and  $46.8$  ng/L EEQs.

For WwTW3, measured concentrations of E1 from the different consignments of effluent collected ranged between  $7.8$  and  $14.5$  ng/L during April 2015 (mean  $11.4 \pm 1.22$  ng/L) and were at their lowest, at between  $< \text{MDL}$  and  $4.7$  ng/L, during July 2015 (mean  $1.9 \pm 0.89$  ng/L). E2 concentrations varied between  $0.4$  and  $4.6$  ng/L (mean  $2.4 \pm 1.08$  ng/L) during April 2015, and were lowest, at  $< \text{MDL}$  during October 2014 and January 2015. Levels of total oestrogenic activity measured at WwTW3 ranged between  $1.6$  ng/L and  $11.0$  ng/L EEQs.

EE2, E2 and E1 were below the minimum detection level in all control water samples. Chemical analysis of the positive control EE2, showed measured concentrations ranging from between 70-130% of the nominal test concentration (mean value,  $7.8 \pm 0.03$  ng/L).



### 3.2 Responses of the ERE-GFP zebrafish to the WwTW effluent exposures

#### 3.2.1 Embryo\_hatching rates (at 72 hpf) and survival rates (at 96 hpf)

All surviving embryos in control groups across all experiments hatched by 72 hpf and this was subsequently used to determine hatching success in other treatments groups. Exposures to EE2 alone resulted in a reduced hatching rate in all experiments conducted (with reduction between 60 and 74%; Fig. 1A-Cii). For the WwTW1 effluent, hatching success for embryos exposed to 100% effluent was reduced ( $p < 0.001$ ) at all sampling periods (Fig. 1Aii) and there were also differences in the hatching rate for the 50% effluent (Fig. 1Aii) during March 2014 (56/90 = 62%,  $p < 0.001$ ) and January 2015 (58/90 = 64%,  $p < 0.01$ ). In the WwTW2 effluent (both 50% and/or 100%) hatching onset was advanced, seen at 48 hpf, at all sampling periods (data not shown). Hatching success for this effluent was reduced in the full-strength effluent at all sampling times (ranging between 28% and 70%, Fig. 1Bii) and in the 50% effluent (Fig. 1Bii) during March 2014 and January 2015 (35/90 = 39%,  $p < 0.001$  and 56/90 = 62%,  $p < 0.001$ , respectively), with an apparent reduction in October 2014 (although this was not statistically significant). In the WwTW3 effluent (50% and 100% effluent), hatching success was reduced at all time points (Fig. 1Cii).

There were no effects on survival for exposures to EE2 (10 ng/L nominal; Fig. 1A-Ci). For effluent exposures, no effects on survival were seen for exposures to the WwTW1 or WwTW3 (Fig. 1Ai, 1Ci), whereas a lower survival rate was observed for embryos exposed to 100 % WwTW2 effluent in March 2014 (45/90 = 50%,  $p < 0.001$ ) and January 2015 (59/90 = 66%,  $p < 0.05$ )(Fig. 1Bi).

### 3.2.2 GFP expression

#### 3.2.2.1 Tissue response patterns at 96 hpf

In some of the control ERE-TG larvae there was a low-level signal of endogenous GFP in the heart (49% of all the larvae) and in the otic vesicle (56% of the larvae), but in most larvae no GFP was observed (Fig. 2A). In embryo larvae exposed to EE2, GFP was observed in the liver, heart, somites, otic vesicle, fins and forebrain (Fig. 2B). Exposure to full-strength (100%) effluent from the three WwTW induced different tissue patterns of the GFP signal (Fig. 2C-E). There was also variation in the GFP detected in body tissues at any one study site for the different effluent collections. In embryos exposed to full-strength WwTW1 effluent, GFP was observed in the liver, heart and somites in March 2014 and heart only at all other sampling-points (Figs 2C & S1C-F) and in the 50% effluent (data not shown).

In embryos exposed to full-strength WwTW2 effluent, GFP was observed in the neuromasts and in the liver, heart and somites in January 2015 (Figs. 2D&S1J), in the heart and somites in March 2014 (Fig. S1G) and in the heart only in July and October 2014 (Figs. S1H&I). In 50% effluent, GFP expression was observed in the liver and somites in January 2015 and in the heart at all other sampling points (data not shown).

For the full-strength WwTW3 effluent, GFP was observed in the heart and somites during January 2015 (Fig. S1L), April 2015 (Fig. S1M) and July 2015 (Fig. 2E) and in the heart only at all other sampling-points (Figs. S1K&N) and 50% effluent exposures. No specific GFP was detected in the liver for this effluent exposures. A time-related analysis of the GFP induction (percentage of embryos with detectable GFP at time points 24 hpf, 48 hpf, 72 hpf and 96 hpf) in the different tissues is shown in the supplementary information (Table S3).

### 3.2.2.2 Tissue GFP quantified at 96 hpf via image analysis

Exposure to 10 ng/L EE2 in all cases resulted in fluorescence induction in the liver, heart and somites, compared with untreated controls, with average fold-increases of 82 in the liver, between no response and 38 in the heart, and between no response and 50 in the somites (Fig. 3A-C). For the WwTW1 effluent, GFP induction was seen in the liver in 100% effluent in March 2014 (16.4-fold,  $p < 0.001$ ; Fig. 3Ai). In the heart, there was a GFP induction in the 50% and 100% effluent treatments during March 2014 (8.4-fold,  $p < 0.05$  and 19.5-fold,  $p < 0.001$ ) and in the 100% effluent treatment in October 2014 (14.5-fold,  $p < 0.01$ ; Fig. 3Aii). There was also GFP induction in the somites for exposure to full-strength effluent in March 2014 (6.07-fold,  $p < 0.05$ ; Fig. 3Aiii).

For the WwTW2 effluent, induction of GFP occurred in the liver in January 2015 for both the 50% and 100% effluent exposures (13.3-fold,  $p < 0.01$  and 61.7-fold,  $p < 0.001$ , respectively Fig. 3Bi) and in the heart for both effluent exposure concentrations at all sampling times (50% effluent – ranging from 10.4 to 13.5-fold;  $p$  values between  $< 0.01$  and  $0.05$ ; 100% effluent – ranging from 11.3 to 19.4-fold;  $p$  values  $< 0.01$ ; Fig. 3Bii). Elevated GFP in the somites occurred in this effluent in January 2015 (both 50%;  $p < 0.01$  and 100% exposures;  $p < 0.001$ ) and during March 2014 (100% effluent  $p < 0.05$ ; Fig. 3Biii).

For the WwTW3 effluent exposures, GFP induction occurred in the heart at all time points with the exception of October 2014 for both effluent exposures (50% effluent – ranging from 11.2 to 15.6-fold;  $p$  values between  $< 0.05$  and  $0.01$ ; 100% effluent – ranging from 10.2 to 20.7-fold;  $p$  values between  $< 0.05$  and  $0.001$ ; Fig. 3Bii; Fig. 3Cii). GFP in the somites was elevated in January and April 2015 (4.9 and 5.6-fold respectively;  $p < 0.05$ ) in 100% effluent exposures only (Fig. 3Ciii).

### 3.2.2.3 Whole-body GFP responses quantified via Western blotting after 96 hpf

Exposure to EE2 (10 ng/L) resulted in whole body elevated GFP for all exposures conducted, compared with untreated controls (39-42-fold-increase,  $p < 0.01$ , Fig. 4). Elevated whole body GFP for the WwTW1 effluent occurred only for the exposure to 50% and full-strength effluent in March 2014, where there was an up to 18-fold induction (Fig. 4A). For exposures to the WwTW2 effluent, GFP was elevated in the full-strength effluent in March 2014 (Fig. 4B; 6-fold increase compared with untreated controls) and both 50% and 100% effluent exposures in January 2015 (Fig. 4; 9-fold increase,  $p < 0.05$ , and 19-fold increase,  $p < 0.05$ , respectively). For the WwTW3 effluent, GFP was elevated only for the exposure to full-strength effluent in April 2015 (10-fold increase compared with controls,  $p < 0.05$ , Fig. 4C).

### 3.2.3 Quantification of whole embryo *vtg* mRNA and *gfp* mRNA

Exposure to EE2 (10 ng/L) resulted in between a 7- and 11-fold-higher relative transcript levels of *gfp* and between 40 and 50 -fold higher relative transcript levels of *vtg*, respectively ( $p < 0.01$ , Fig. 5A-C). In the WwTW1 effluent exposures, *gfp* mRNA levels appeared to be higher (approximately 3-fold induction) in embryos exposed to full-strength effluent during March 2014, but this was not statistically significant (Fig. 5Ai). A higher level of *vtg* mRNA level occurred only in embryos exposed to full-strength WwTW1 effluent during March 2014 (8-fold higher compared to controls,  $p < 0.01$ , Fig. 5Aii), and this was higher also when compared with levels of *vtg* mRNA levels measured in July 2014, October 2014 and January 2015 ( $p < 0.05$  Fig. 5Aii). *gfp* mRNA expression in the WwTW2 effluent exposures was higher compared with controls for the full-strength effluent in January 2015 (a 5-fold induction;  $p < 0.05$ , Fig. 5Bi) and compared also with the full-strength effluent in July and October 2014 ( $p <$

0.05, Fig. 5Bi). In the full-strength WwTW2 effluent, the *vtg* mRNA level in embryo larvae was higher in both March 2014 and January 2015 (3-fold and 22-fold induction, respectively, both  $p < 0.01$ ; Fig. 5Bii) compared with controls. In the full-strength effluent exposures, *vtg* mRNA levels were higher in January 2015 compared with all other time-points ( $p < 0.05$ , Fig. 5Bii). For the WwTW3 effluent, the only indications of a higher amount of *gfp* mRNA expression (approximately 2-fold) was for the full-strength effluent exposures in January and April 2015, but these were not statistically significant (Fig. 5Ci). *vtg* mRNA levels were higher in embryos following exposure to full-strength effluent during April 2015 only compared with controls (5-fold higher,  $p < 0.05$ , Fig. 5Cii) and compared with full strength effluent for the October 2014 and January and July 2015 exposures ( $p < 0.05$ , Fig. 5Cii).

The magnitude of responses for *vtg* induction was higher compared to *gfp* in both the EE2 (Fig 6A) and wastewater effluent exposures (Fig. 6B). For the data combined there was a positive correlation between the levels of *gfp* expression and the levels of *vtg* expression for the EE2 exposure ( $r^2 = 0.5$ ;  $p < 0.01$ ;  $n = 15$  fish per treatment) whereas a very weak relationship was found for the effluent exposures ( $r^2 = 0.2$ ,  $p < 0.05$ ;  $n = 15$  fish per treatment) (Fig 6).

## 4. Discussion

### 4.1 Temporal variation in effluent oestrogenicity

Measured concentrations of the steroidal oestrogens E1 and E2 (and ratios between them) in the three WwTW effluents varied by more than 3-fold over the 12-month period supporting previous reports on temporal variation in the oestrogenic content of wastewater effluents (Vajda et al., 2008; Zhou et al., 2012). Relatively high

levels of E1, in comparison with E2, were measured at all three treatment works (here ranging from 0.03 - 36.7 ng/L), and were analogous with those that have reported previously in WwTW effluents (Furtula et al., 2012; Williams et al., 2012). The higher levels of E1 are explained by a relatively slower degradation rate of E1 and by the oxidation of E2 to E1 that occurs during wastewater treatment processes (Combalbert and Hernandez-Raquet, 2010; Desmiarti and Li, 2013). There was no obvious temporal variability in the synthetic oestrogen EE2 at any of the studied treatment works, with measured concentrations almost consistently below the method detection limit (0.5 ng/L); these levels also compare favourably with other studies internationally (e.g. Cui et al., 2006; Hannah et al., 2009).

Various studies have shown that higher temperatures in the summer months can enhance the efficiency of biological treatment processes in removing steroidal oestrogens, due to increased growth (and hence biodegradation) rates of microbial communities, with the reverse occurring during the winter (Jin et al., 2008; Martinović et al., 2008), but we did not find this as a consistent pattern for the different steroid oestrogens across the different WwTWs. The efficiency of a WwTW in removing both natural and synthetic oestrogens, however, is influenced by many different factors including temperature and rainfall, as well as microbial content, all of which can vary seasonally. It is also the case that high temperatures, in combination with lower rainfall, can result in microbial communities operating at a much lower efficiency rate (Cruikshank and Gilles, 2007).

We found lower levels of steroidal oestrogens in WwTW effluents were associated with higher rainfall levels when they occurred during the summer months. Higher levels of rainfall (and hence greater dilution) have been shown previously to lead to lower oestrogenic content in WwTW effluents in other studies (Diniz et al., 2005;

Phillips et al., 2012). Contrasting with this however, high levels of rainfall (and hence higher flow rates of influent entering a WwTW) can also result in a reduced total Hydraulic Retention Time (HRT), thus leading to reduced treatment efficiencies (Kumar et al., 2015; Phillips et al., 2012). This may account for the high level of E1 observed at WwTW2 during January 2015 (i.e. 46.8 ng/L EEQs) when there was a period of very high rainfall. This WwTW also had a relatively short HRT (only 2.5 hrs) compared with WwTW1 (18 hrs) and WwTW3 (12 hrs) and a longer HRT is generally associated with a more efficient removal of steroidal oestrogens (Ejhed et al., 2018; Huang et al., 2008). Other factors that may have contributed to the variability in the oestrogenic activity in the different WwTW effluents include fluctuations in the input of organic matter (quantified by biological oxygen demand [BOD] and total suspended solids [TSS]), that affect the binding of environmental oestrogens and thus their bioavailability for uptake.

Whilst seasonal influences of temperature and rainfall are important factors in determining levels of steroidal oestrogens in wastewater effluent, the method of treatment employed at a WwTW can be extremely influential. Our study suggests that WwTW1, with an Activated Sludge (AS) secondary treatment process, was more efficient at removing steroidal oestrogens compared with the Biological Filter (BF) processes in place at the other WwTWs which concurs with other observations on removal efficiency between the two processes (Gabet-Giraud et al., 2010; Johnson et al., 2007).

Chemical make-up of the wastewater influent to a treatment works can also affect the subsequent effluent quality. In this study, the majority of wastewater entering all three WwTWs was domestic in origin, with only a small percentage of industrial input (between 3.3 to 7.1%). We found no link between the total population equivalent size

of a particular WwTW and the amount of oestrogenic activity in the resulting effluent. Some of the seasonal variations in the oestrogen content of the effluents at WwTW1 and WwTW2 could be due to changes in the seasonal resident population (e.g. due to student enrolment for local universities) and which may contribute to the higher oestrogenic content observed at both works during March 2013 and January 2014. The variability in the oestrogenic content of the WwTW effluents emphasises the importance of avoiding extrapolations from single time point analysis.

#### *4.2 Responses of the ERE-GFP zebrafish embryo-larvae to the effluent treatments*

##### *4.2.1 Survivorship and Hatching*

Exposure to EE2 (at 10 ng/L) had no effects on survivorship supporting the findings from other studies (Jackson and Klerks, 2019; Schäfers et al., 2007). The enhanced mortality seen at 96 hpf for exposures to full strength WwTW2 effluent during March 2014 and January 2015 corresponded with a high oestrogenic potency but this is unlikely responsible for these mortalities. Rather this is likely due to some other physio-chemical features of the effluent at these times, which might include the presence of toxic contaminants, or elevated ammonia levels due to poor treatment efficiency. At these sampling times, levels of both chloride and ammonia (up to 4000 and 40 mg/L, respectively) were known to be elevated caused by saline infiltration from the local sewerage system. Reduced hatching rates in the EE2 and effluent exposures are in accordance with that seen in published studies (Smolders et al., 2002; Versonnen and Janssen, 2004). The mechanism(s) for this effect are not known but they may include chemicals in the effluent affecting hatching enzyme systems as had been reported for selected chemicals previously (De la Paz et al., 2017; Trikić et al., 2011).



#### 4.2.2 GFP and VTG expression

Exposure to EE2 induced GFP responses in the ERE-GFP transgenic zebrafish embryo larvae in a very wide range of tissues, including heart, liver, somite muscle and forebrain, in agreement with other studies (Green et al., 2016; Lee et al., 2012; Moreman et al., 2017). The wide range of target tissues for EE2 together with its high potency and its tendency to bio-concentrate in body tissues in fish (Fenlon et al., 2010; Yang et al., 2014) serve to re-enforce the key role this synthetic oestrogens likely plays in the feminised responses seen in wild fish populations in UK rivers (Tyler and Jobling, 2008).

Induction of GFP in the ERE-GFP transgenic zebrafish exposed to the WwTW effluents were seen consistently in the heart, liver, muscle somites and otic vesicle. Responses were seen also in the forebrain and neuromasts for exposure to the WwTW2 effluent. All of these tissues have been shown previously to be responsive to steroidal oestrogens but also to other environmental oestrogens including 4-nonylphenol, and various bisphenolic compounds (Green et al., 2016; Lee et al., 2012; Moreman et al., 2017), that are known to occur commonly in WwTW effluents (Hu et al., 2019; Klečka et al., 2010). In the WwTW2 effluent in January 2015, the strong GFP induction in the liver was most likely a consequence of exposure to high level of E2 at this time. In contrast, in the WwTW3 effluent exposure in April 2015, there was little or no GFP expression in the liver, but induction was found in the somites, suggesting a response mediated by a non-steroidal oestrogen, with possible chemical candidates for this such as nonylphenol or bisphenolic compounds. The variation in tissue responses indicate the potential for differing health effects between different WwTW effluents and even for a single effluent over different times in the year. The differences in tissue specificities for the GFP induction may be due to differences in

tissue expression and interactions with the three oestrogen receptor subtypes and other related receptors that can form heterodimers with ERs (Gorelick et al., 2014; Tingaud-Sequeira et al., 2004). The high sensitivity of the heart to both EE2 and oestrogens contained in WwTW effluents is consistent with responses seen in another oestrogen responsive transgenic zebrafish line (Gorelick et al., 2014) and serves to further illustrate the need for further studies on possible health outcomes in fish for chronic environmental exposures given the known crucial role of oestrogenic signalling in heart development (Celeghin et al., 2011; Pugach et al., 2016) and findings for harmful effects on heart development in fish (Brown et al., 2019; e.g. Moreman et al., 2017).

*vtg* mRNA levels in the ERE-GFP fish mirrored the concentrations of steroidal oestrogens measured for exposure all three WwTW effluents, supporting steroidal oestrogens being largely responsible for *vtg* induction in fish exposed to wastewater effluents; E1 and E2, together with EE2, often make up 80% of a WwTW effluent oestrogenic activity (Desbrow et al., 1998; Rodgers-Gray et al., 2000). There was a positive correlation between *gfp* mRNA levels and *vtg* mRNA levels for the EE2 exposures but this was less apparent in the case of WwTW effluent exposures. For the latter, this may be because the response for GFP (whole body) measures responses across many tissues and induction/effect mechanisms compared with that for VTG that occurs in the liver only. Direct comparison of whole embryo-larvale *gfp* mRNA expression with *vtg* mRNA expression demonstrated that *vtg* mRNA expression was more responsive in terms of the sensitivity threshold for induction and magnitude of response; the huge dynamic range for *vtg* mRNA induction (and its protein product of up to a million-fold; Tyler et al., 1996) is well established.

## Conclusion

This study demonstrates that the ERE-GFP transgenic zebrafish model can be applied to detect environmental oestrogens in wastewater effluent samples and exhibits tissue-specific effects similar to those seen for exposure to different individual environmental oestrogens. Differences in GFP tissue patterning (and of other biological endpoints) were found to be reflective of the varying concentrations of steroidal oestrogens found in the different wastewaters (both temporally and between treatment works), and illustrated the considerable dynamic nature of the oestrogenic potencies of the WwTW effluents. Currently, analytical chemistry is generally the method of choice in large and regular chemical monitoring campaigns but this approach largely ignores the combined effects of substances with the same or similar mode of action. The ERE-GFP transgenic zebrafish model, in contrast, provides an integrative measure for the effects of exposure to oestrogenic chemical mixtures (here, in wastewater effluents) and in turn provides a highly complementary approach with analytical chemistry for chemical monitoring. Whilst these results suggest that GFP induction is less sensitive as a detection method than VTG for detecting responses to oestrogen, the ERE-GFP zebrafish provides an integrative model system to help understand more fully the tissues targeted for more effective health effects analyses, and thus ecological risk assessment posed by wastewater effluents, specifically in respect of AOP frameworks.

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#### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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**Table 1. Details for the three WwTWs and physiochemical data for their discharged effluents.**

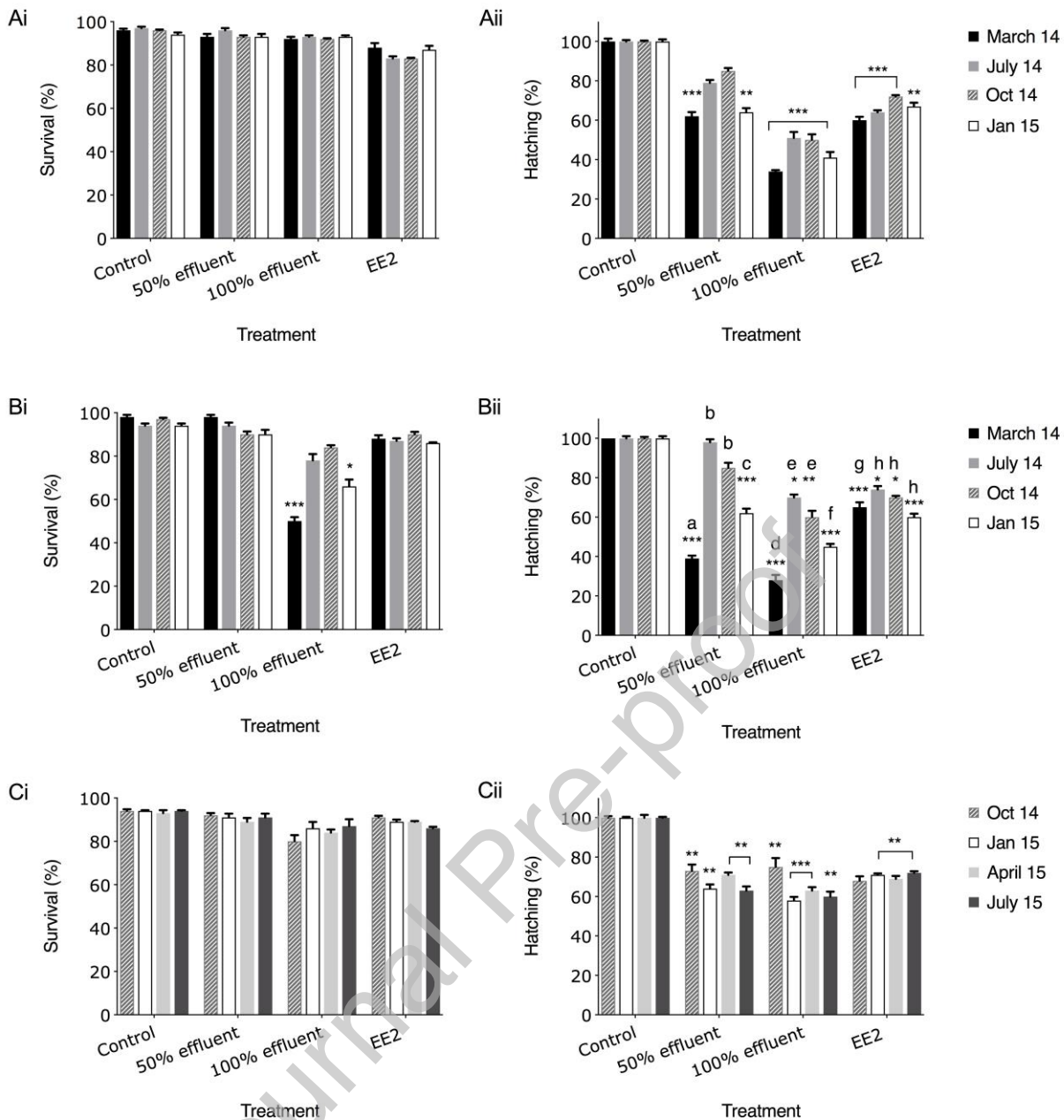
Details include the treatment processes (AS = activated sludge, BF = biological filter, BAFF = biological aerated flooded filter) used at each treatment works (including total hydraulic retention times [HRT], sludge retention times [SRT]), total population equivalent values for each site and types of industrial discharges. Data also include concentrations of the major steroidal oestrogens (E1, E2 and EE2) measured in the test effluents collected from the WwTW study sites during all experimental periods and

calculated oestradiol equivalents (EEQs). Data are reported as mean  $\pm$  SE (n = 3 measurements per physiochemical parameter).

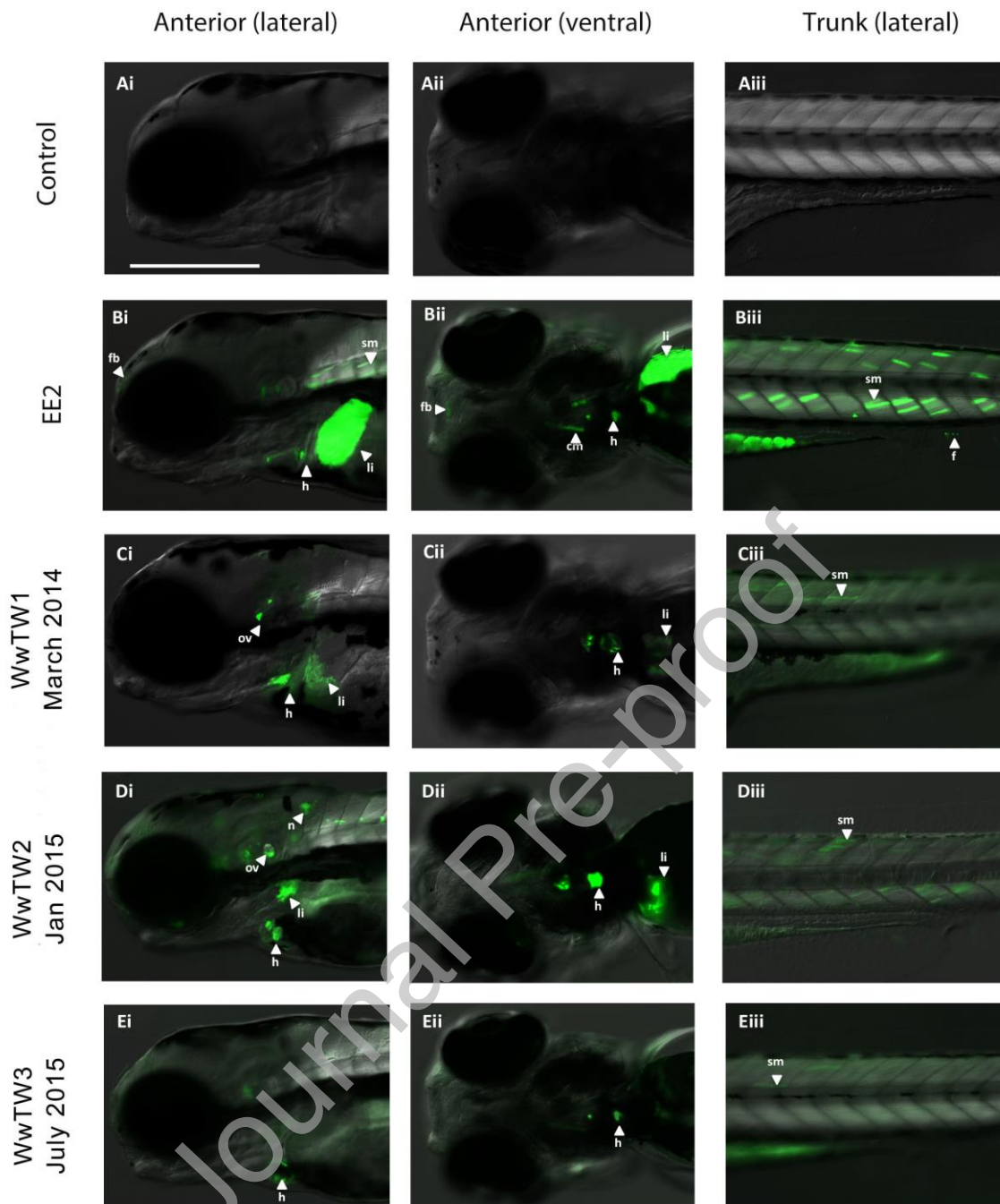
Site	WwTW1				WwTW2				WwTW3			
Total population equivalent	141698				117574				23648			
Industrial population equivalent	4713				6100				1670			
Proportion of industrial wastewater (%)	3.3				5.2				7.1			
Secondary treatment	AS/BF				BAFF				BF			
Tertiary treatment	Nitrifying BAFF UV Disinfection				UV Disinfection				None			
Total HRT (hrs)	6 AS/12BF				2.5				12			
Total SRT (days)	6				n/a				n/a			
Number of permitted industrial discharges	49				30				<10			
Types of industrial/trade waste	Car washes Plant wash down Laundrettes Rendering plant Electroplater University of Exeter Cattle market Gas holders Swimming pools Food processing				Car washes Plant wash down Laundrettes Dockyard Fish processor Waste oil processor Gas holders Swimming pools Recycling plant				Car washes Plant wash down Laundrettes Brewery Tip leachate			
Physicochemical parameter	March 2014	July 2014	October 2014	January 2015	March 2014	July 2014	October 2014	January 2015	October 2014	January 2015	April 2015	July 2015
pH	6.5 $\pm$ 0.07	7.0 $\pm$ 0.05	7.0 $\pm$ 0.06	7.2 $\pm$ 0.10	7.3 $\pm$ 0.05	6.9 $\pm$ 0.06	7.2 $\pm$ 0.05	7.3 $\pm$ 0.09	7.4 $\pm$ 0.05	7.7 $\pm$ 0.09	7.5 $\pm$ 0.05	7.6 $\pm$ 0.03 20.3
Rainfall (mm)	0.0	0.0	15.4 $\pm$ 1.45	13.1 $\pm$ 1.2	0.0	5.3 $\pm$ 6.2	32.3 $\pm$ 16.5	16.9 $\pm$ 4.7	32.0 $\pm$ 13.5	16.5 $\pm$ 4.7	0.0	12.6
Suspended solids (mg/L)	24 $\pm$ 0.76	15 $\pm$ 1.45	15 $\pm$ 1.61	7 $\pm$ 0.94	21 $\pm$ 1.21	9 $\pm$ 0.93	14 $\pm$ 1.57	23 $\pm$ 1.24	9 $\pm$ 0.83	21 $\pm$ 1.23	16 $\pm$ 1.37	10 $\pm$ 1.69
BOD (mg/L)	6 $\pm$ 0.87	11 $\pm$ 0.52	7 $\pm$ 0.34	3 $\pm$ 0.76	11 $\pm$ 0.58	7 $\pm$ 0.23	12 $\pm$ 0.18	6 $\pm$ 0.44	13 $\pm$ 0.92	9 $\pm$ 0.67	7 $\pm$ 0.26	13 $\pm$ 0.58
COD (ng/L)	63 $\pm$ 0.52	74 $\pm$ 0.77	56 $\pm$ 0.62	23 $\pm$ 0.91	67 $\pm$ 0.25	80 $\pm$ 0.69	70 $\pm$ 0.87	53 $\pm$ 0.64	54 $\pm$ 0.88	48 $\pm$ 0.64	55 $\pm$ 0.72	45 $\pm$ 0.82
NH <sub>3</sub> (mg/L)	2 $\pm$ 0.18	8 $\pm$ 0.06	6 $\pm$ 0.10	8 $\pm$ 0.07	n/a	n/a	n/a	n/a	1.6 $\pm$ 0.16	0.4 $\pm$ 0.07	0.2 $\pm$ 0.05	1.2 $\pm$ 0.14
Cl <sup>-</sup> (mg/L)	n/a	n/a	n/a	n/a	1491 $\pm$ 41.7	2454 $\pm$ 31.1	3189 $\pm$ 55.4	1777 $\pm$ 59.6	n/a	n/a	n/a	n/a
E1 (ng/L) <sup>a</sup>	16.7 $\pm$ 1.11	0.6 $\pm$ 0.24	4.4 $\pm$ 0.2	0.6 $\pm$ 0.04	9.0 $\pm$ 0.97	3.9 $\pm$ 1.00	1.1 $\pm$ 0.42	36.7 $\pm$ 6.05	2.2 $\pm$ 1.03	5.3 $\pm$ 1.95	11.4	1.9 $\pm$ 0.89
E2 (ng/L) <sup>a</sup>	1.2 $\pm$ 0.12	1.6 $\pm$ 0.42	<MDL	<MDL	4.0 $\pm$ 0.09	1.2 $\pm$ 0.07	<MDL	*	<MDL	<MDL	1.08	0.16
EE2 (ng/L) <sup>a</sup>	<MDL	L	<MDL	<MDL	<MDL	<MDL	<MDL	0.08 $\pm$ 0.09	<MDL	<MDL	L	<MDL
EEQs (ng/L)	13.7 $\pm$ 0.73	2.1 $\pm$ 0.54	3.30 $\pm$ 0.15	0.6 $\pm$ 0.03	10.7 $\pm$ 0.73	4.1 $\pm$ 0.69	1.1 $\pm$ 0.31	46.8 $\pm$ 9.34	1.6 $\pm$ 0.77	5.7 $\pm$ 1.46	11.0	2.6 $\pm$ 0.61

<sup>a</sup> The method detection limits (MDL) for E1, E2 and EE2 were 0.2 ng/L, 0.4 ng/L and 0.5 ng/L,

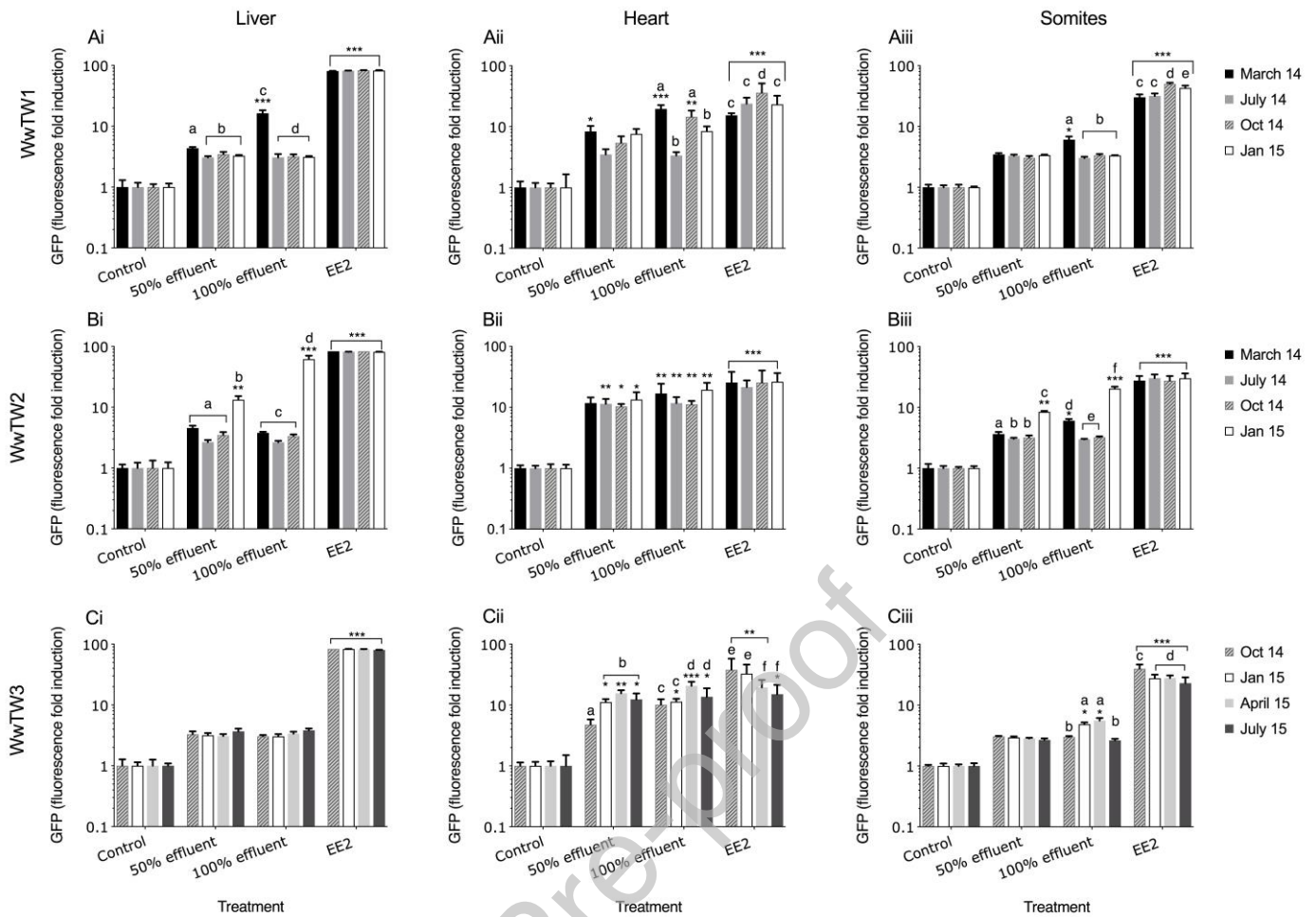
respectively and the method quantification limits (MQL) for E1, E2 and EE2 were 0.6 ng/L, 1.2 ng/L and 1.5 ng/L, respectively. To calculate mean oestrogen concentrations, concentrations that were over the limits of detection ( $\geq$  MDL) but below the limits of quantification ( $<$  MQL) were assigned the MDL value. Concentrations below the MDL were considered to be zero. Individual measured concentrations of E1 and E2 are presented in Supplementary Table S3. EE2 concentrations were below the MDL (0.5 ng/L) throughout. \* samples did not pass the quality control check and the qualifier ion could not be detected.



**Fig. 1. Hatching rates (at 72 hpf) and subsequent survival (at 96 hpf) in ERE-GFP zebrafish embryos.** Transgenic zebrafish embryos in control (unexposed), EE2 (10 ng/L, positive control) and effluent (50% or 100%) from (A) WwTW1, (B) WwTW2 or (C) WwTW3. Effluent exposures were conducted using 24-hr composite wastewater effluent samples collected at 4-time points over a 12-month period. Exposures were conducted in triplicate for any given effluent collection. Data are reported as mean  $\pm$  SE (n=15 fish per treatment). Asterisks denote a significant difference (\* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001) compared with controls. Different letters denote a significant difference within treatment groups ( $p$  < 0.05).

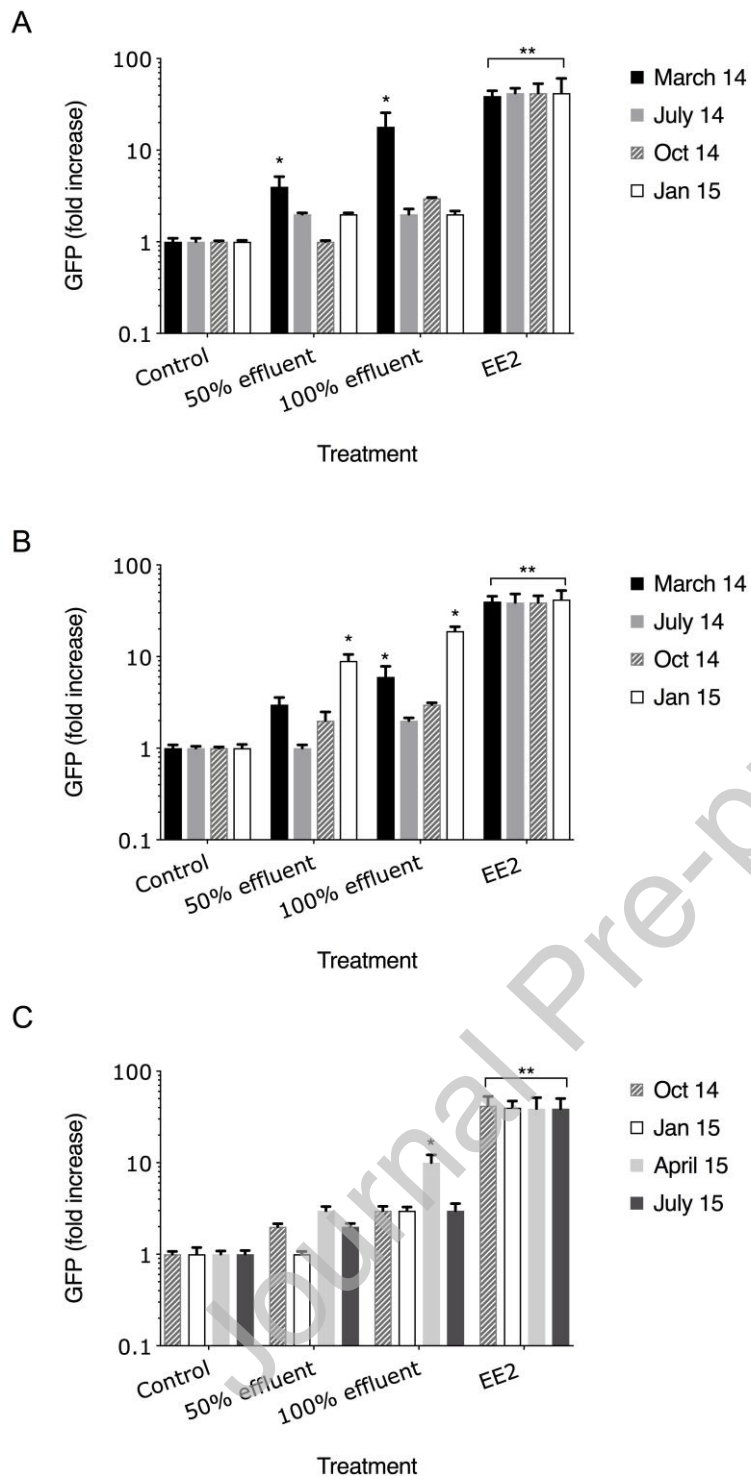


**Fig 2. GFP in ERE-GFP zebrafish embryo-larvae exposed to three different wastewater effluents from fertilisation to 96 hours post fertilisation (hpf).** Transgenic zebrafish embryo-larvae in control (unexposed) (A), EE2 (10 ng/L, positive control) (B), and wastewater effluent (100%) exposed groups from WwTW1 (C), WwTW2 (D) and WwTW3 (E). Effluent exposures were conducted using 24-hr composite wastewater effluent samples collected at 4-time points over a 12-month period. GFP detection is shown for regions in the head (lateral (A-Ei) and ventral (A-Eii views) and trunk (lateral view A-Eiii). cm=cranial muscles; f=fin; fb=forebrain; h= heart; li=liver; n=neuromast; ov=otic vesicle; sm=somite muscles. Bar = 200  $\mu$ m.

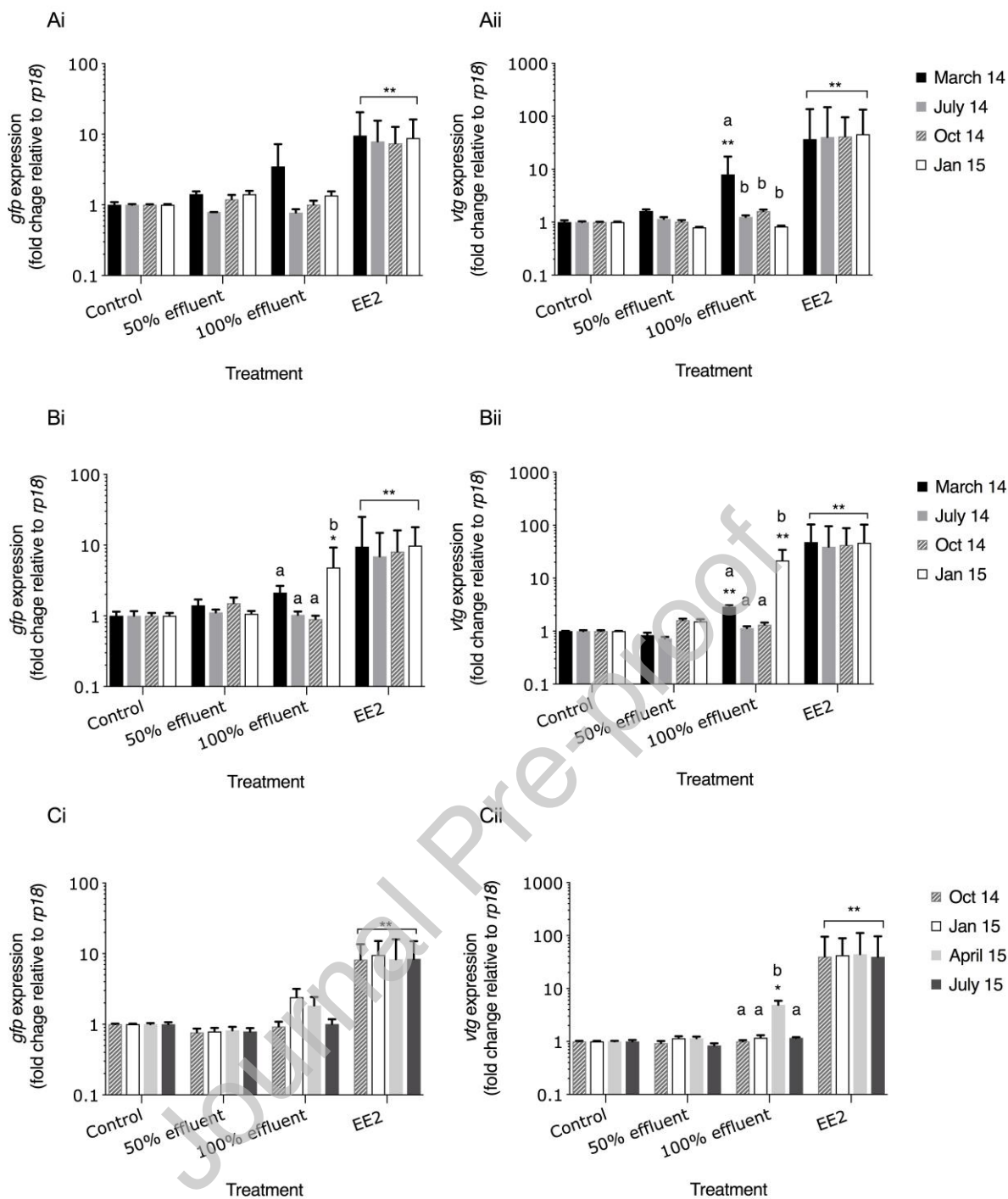


**Fig 3. Quantification of GFP in target tissue responses in 96 hpf ERE-GFP zebrafish exposed to three different wastewater effluents, as determined by fluorescence induction.** Data show fold-increase in GFP levels relative to controls in the liver (i), heart (ii) and somites (iii) in ERE-GFP larvae exposed to EE2 (10 ng/l or WwTW effluent (50% or 100%) from WwTW1(A), WwTW2 (B) and WwTW3 (C). Effluent exposures were conducted using 24-hr composite wastewater effluent samples collected at 4-time points over a 12-month period. Exposures were conducted in triplicate for any given effluent collection. Data are reported as mean  $\pm$  SE ( $n = 15$  fish per treatment). Asterisks denote a significant difference (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) compared with controls. Different letters denote a significant difference within treatment groups ( $p < 0.05$ ).



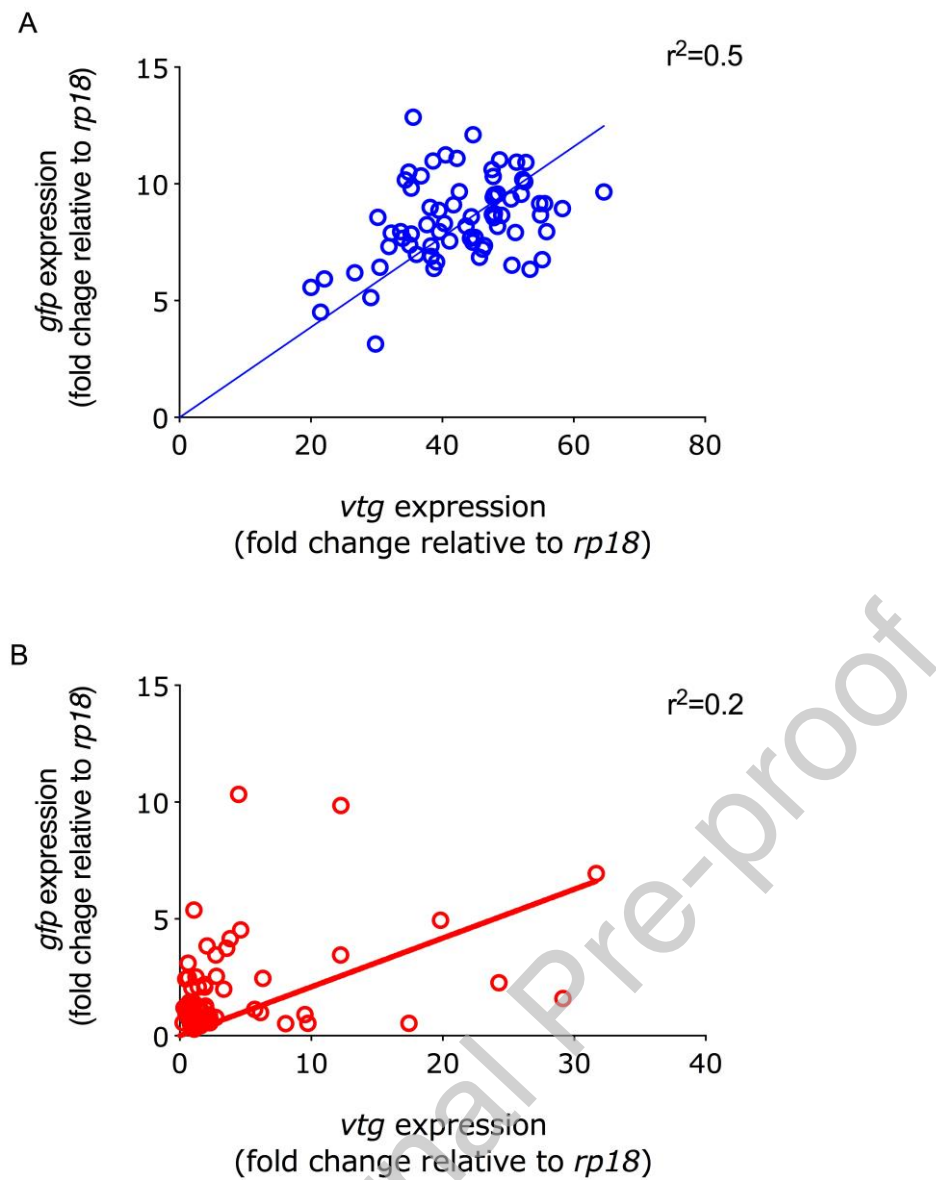


**Fig 4. Relative GFP levels in whole ERE-GFP zebrafish embryos exposed to selected WwTW effluents and EE2, as quantified through Western blotting.** Data show fold increase in GFP expression compared with controls following exposure to EE2 (10 ng/l) or WwTW effluent (50% or 100%) from WwTW1 (A), WwTW2 (B) and WwTW3 (C). Effluent exposures were conducted using 24-hr composite wastewater effluent samples collected at 4-time points over a 12-month period. Exposures were conducted three times for any given effluent collection. Data are reported as mean  $\pm$  SE (n = 15 fish per treatment). Asterisks denote a significant difference (\* $p$  < 0.05, \*\* $p$  < 0.01) compared with controls.



**Fig. 5. *gfp* and *vtg* mRNA levels in whole ERE-GFP zebrafish embryo-larvae.** Data show average fold-changes (normalised against the expression of the control gene ribosomal protein L8 (*rp18*)) compared with controls following exposure to EE2 (10 ng/l) or WwTW effluent (50% or 100%) for WwTW1 (Ai-ii), WwTW2 (Bi-ii) and WwTW3 (Ci-ii). Effluent exposures were conducted using 24-hr composite wastewater effluent samples collected at 4-time points over a 12-month period. Exposures were conducted in triplicate for any given effluent collection. Data are reported as means  $\pm$  SE ( $n = 15$  fish per treatment). Asterisks denote a significant difference ( $*p < 0.05$ ,  $**p < 0.01$ ) compared with treatment controls at the relevant time point. Different letters denote a significant difference within groups ( $p < 0.05$ ).





**Fig 6. Correlation between *gfp* mRNA and *vtg* mRNA levels in ERE-GFP zebrafish whole embryos.** Data show (A) EE2 (10 ng/l, positive control) treatment groups (n = 15 fish) and (B) effluent (50% & 100% combined; n = 15 fish per treatment) treatments groups for the three WwTWs. Effluent exposures were conducted using 24-hr composite wastewater effluent samples collected at 4-time points over a 12-month period. Exposures were conducted in triplicate for any given effluent collection.