

## Supplementary Information

### 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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#### Supplementary Material and Methods

Protocol for LC-MS used for detection of the steroidal oestrogens	Page S2
<b>Table S1.</b> Retention times and MRM conditions used for UHPLC–ESI-MS/MS analysis of steroidal oestrogens	Page S4
<b>Table S2.</b> Overview of RT-qPCR parameters	Page S5
<b>Table S3.</b> Survival rates in ERE-GFP zebrafish embryos exposed from 0-96 hpf	Page S6
<b>Table S4.</b> GFP levels in ERE-GFP zebrafish embryos exposed from 0-96 hpf	Page S7
<b>Figure S1.</b> Tissue GFP levels in ERE-TG zebrafish embryos exposed from to 0-96 hpf at 4 timepoints during the year	Page S8
<b>Supplementary References</b>	Page S9

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## Supplementary Material and Methods

### ***SPE protocol and quantification of steroidal estrogens in the WwTW effluent using LC-MS.***

To perform the cleaned up using DSC-NH<sub>2</sub> cartridges according to Flores et al. (2008), 0.25 mL of the dried Oasis HLB extracts were first dissolved in 1 mL of ethyl acetate. DSC-NH<sub>2</sub> cartridges were then conditioned with 1 mL of ethyl acetate and 1 mL of ethyl acetate/methanol (4:1, v/v). The extract was loaded onto the cartridge and the eluate was directly collected; the cartridges were further rinsed with 2 mL of the solvent mixture and the final extracts were taken to dryness. After this, extracts were reconstituted in 100 µL of water/acetonitrile (7:3, v/v) and passed through 0.22 µm centrifuge filters and stored at -80°C before analyses. Ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analyses were carried out using a Waters Acquity UHPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). Samples were separated using a reverse phase Acquity UHPLC BEH C18 column (1.7 µm particle size, 2.1 mm × 100 mm, Waters, Manchester, UK) fitted with an Acquity UHPLC BEH C18 VanGuard pre-column (130 Å, 1.7 µm, 2.1 mm × 5 mm, Waters, Manchester, UK) maintained at 25 °C. Injection volume was 20 µL and mobile phase solvents were 94.95% water, 5% MeOH, 0.05% ammonium hydroxide (A) and 99.95% MeOH, 0.05% ammonium hydroxide in an initial ratio (A:B) of 40:60. Separation was achieved using a flow rate of 0.15 mL/min with the following gradient: 40:60 to 20:80 in 10 min; then from 20:80 to 0:100 and held for 5 min, then return to initial condition at 15 min and equilibration for 7 min. Retention times, ionisation and fragmentation settings are reported in Table 1. MS/MS was performed in the Multiple Reaction Mode (MRM) using ESI in the negative mode and two characteristic fragmentations of the

deprotonated molecular ion  $[M-H]^-$  were monitored. The first and most abundant one was used for quantitation, while the second one was used as a qualifier. Other parameters were optimised as follows: capillary voltage  $-3.13$  kV, extractor voltage  $8$  V, multiplier voltage  $650$  V, source temperature  $120$  °C, desolvation temperature  $400$  °C. Argon was used as collision gas (P collision cell:  $3 \times 10^{-3}$  mbar), while nitrogen was used as both the nebulizing ( $100$  L/h) and desolvation gas ( $600$  L/h). Mass calibration of the spectrometer was performed with sodium iodide. Data were acquired using MassLynx 4.1 and the quantification was carried out by calculating the response factor of estrogenic compounds to internal standards. Concentrations were determined using a least-square linear regression analysis of the peak area ratio versus the concentration ratio native to deuterated. At least, five point calibration curve ( $R^2 > 0.99$ ) covering the range of concentrations for all compounds were used within the linear range of the instrument.

**Table S1.** Retention times and MRM conditions used for UHPLC–ESI-MS/MS analysis of 17 $\alpha$ -ethinyloestradiol (EE2), 17 $\beta$ -oestradiol (E2) and oestrone (E1) in negative mode.

Compound	Retention time		MRM transitions		TQ parameters	
		Precursor	qualification ion	quantification ion	Cone (V)	Collision (V)
EE2	5.01	295	145	159	50	40
E2	5.08	271	145	183	50	45
E1	5.06	269	145	143	50	45
EE2-d4	4.97	299	147	161	50	50
E2-d4	5.04	275	147	187	50	50
E1-d4	5.04	273	145	147	50	45

**Table S2.** Overview of RT-qPCR parameters

<b>Gene name</b>	<b>Gene symbol</b>	<b>Forward primer (5'-3')</b>	<b>Reverse primer (5'-3')</b>	<b>Ta (°C)</b>	<b>Efficiency (%)</b>	<b>Reference</b>
Vitellogenin 1	<i>vtg1</i>	CATCTTATGCTGGTGATTTGTC	GTTAATTGCAGTACAGCAGTGG	62.0	110	(Lange et al., 2012)
Ribosomal protein L8	<i>rpl8</i>	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	59.5	102	(Santos et al., 2007)
Enhanced green fluorescent protein	<i>egfp</i>	CGACGGCAACTACAAGAC	TAGTTGTA CTCCAGCTTGTGC	60.0	96.5	(Cooper et al., 2021)

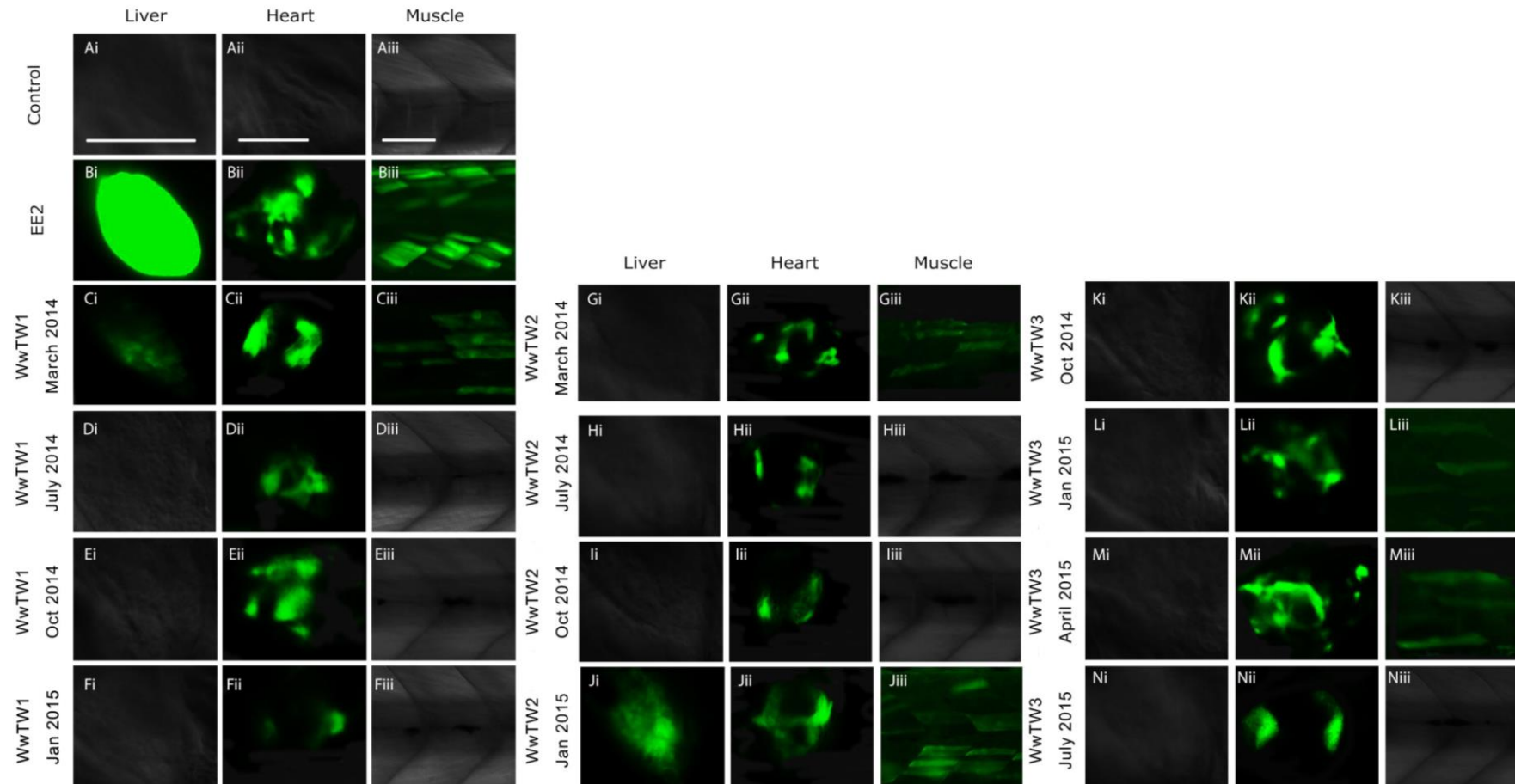
**Table S3.** Survival rates (%) in either control (unexposed), WwTW effluent exposed or EE2 exposed ERE-GFP zebrafish (exposed embryos from 0-96 hours post fertilisation; hpf). Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. Exposures were conducted in triplicate for any given effluent collection. Data are reported as mean (n = 45 fish per treatment).

	WwTW1				WwTW2					WwTW3			
	Control	50% effluent	100% effluent	EE2	Control	50% effluent	100% effluent	EE2		Control	50% effluent	100% effluent	EE2
	% number of embryos				% number of embryos					% number of embryos			
<b>March 2014</b>									<b>October 2014</b>				
24hpf	97	93	94	91	100	100	98	93	24hpf	98	96	91	93
48hpf	96	93	93	88	99	99	79	90	48hpf	94	94	84	92
72hpf	96	93	93	88	99	98	50	88	72hpf	94	92	79	91
96hpf	96	93	92	88	98	98	50	88	96hpf	94	92	79	91
<b>July 2014</b>									<b>January 2015</b>				
24hpf	97	98	96	88	96	96	91	93	24hpf	94	93	93	92
48hpf	97	96	93	86	94	94	82	89	48hpf	94	92	89	90
72hpf	97	96	93	84	94	94	78	87	72hpf	94	91	87	89
96hpf	97	96	93	83	94	94	78	87	96hpf	94	91	86	88
<b>October 2014</b>									<b>April 2015</b>				
24hpf	98	98	94	92	97	96	90	95	24hpf	96	93	92	94
48hpf	96	93	92	86	97	91	87	91	48hpf	94	90	88	91
72hpf	96	93	92	84	97	90	84	90	72hpf	94	89	87	89
96hpf	96	93	92	84	97	90	84	90	96hpf	93	89	84	89
<b>January 2015</b>									<b>July 2015</b>				
24hpf	97	94	96	90	97	92	73	94	24hpf	95	94	94	92
48hpf	95	94	95	87	96	89	69	88	48hpf	94	92	89	92
72hpf	94	93	93	87	94	86	66	86	72hpf	94	91	87	90
96hpf	94	93	93	87	94	86	66	86	96hpf	94	91	87	90

**Table S4.** GFP in either control (unexposed), WwTW effluent exposed or EE2 exposed ERE-GFP zebrafish embryos (exposed from 0-96 hours post fertilisation; hpf). Data show percentage of ERE-GFP embryos (n = 45 per treatment), expressing GFP in the heart, liver, brain, somites and otic vesicle for all exposure treatments for the three WwTWs. Effluent exposures were conducted using 24-hour 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.

Site	WwTW1				WwTW2				WwTW3			
Tissue Type	24 hpf	48 hpf	72 hpf	96 hpf	24 hpf	48 hpf	72 hpf	96 hpf	24 hpf	48 hpf	72 hpf	96 hpf
<b>Control</b>	% number of embryos				% number of embryos				% number of embryos			
Liver	0	0	0	0	0	0	0	0	0	0	0	0
Heart	0	23	31	43	0	23	42	52	0	25	50	52
Brain	0	0	0	0	0	0	0	0	0	0	0	0
Somites	0	0	0	0	0	0	0	0	0	0	0	0
Otic vesicle	0	23	54	58	0	23	57	59	0	24	53	52
<b>50% effluent</b>												
Liver	0	0	0	0	0	0	0	0	0	0	0	0
Heart	0	22	53	58	0	25	47	61	0	26	55	58
Brain	0	0	0	0	0	0	0	0	0	0	0	0
Somites	0	0	0	0	0	0	0	0	0	0	0	0
Otic vesicle	0	25	59	61	0	28	65	59	0	25	54	54
<b>100% effluent</b>												
Liver	0	0	15	17	0	8	25	25	0	0	0	0
Heart	0	26	89	87	0	31	100	100	0	29	91	96
Brain	0	0	0	0	0	0	0	0	0	0	0	0
Somites	0	0	16	18	0	0	42	42	0	4	21	25
Otic vesicle	0	27	59	64	0	33	66	63	0	28	57	58
<b>EE2</b>												
Liver	55	100	100	100	57	100	100	100	48	100	100	100
Heart	54	89	100	100	52	90	100	100	45	92	100	100
Brain	0	0	21	57	0	0	27	63	0	65	67	69
Somites	61	100	100	100	63	100	100	100	0	0	31	62
Otic vesicle	0	65	64	70	0	68	68	69	0	65	67	69

**Figure S1.** GFP in the liver, heart and somite muscle in ERE-TG zebrafish embryos exposed to selected wastewater effluents from fertilisation to 96 hours post fertilisation at 4 timepoints during the year. Transgenic zebrafish embryos in control (unexposed) (A), EE2 (10 ng/L, positive control) (B), and wastewater effluent (100%) exposed groups at WwTW1 (C-F), WwTW2 (G-J) and WwTW3 (K-N). Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. Exposures were conducted three times for any given effluent collection. GFP expression is shown (lateral view) in the liver (i) and somite muscle (iii), and in the heart (ii, ventral view). Bars = 100  $\mu$ m.





## Supplementary References

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