



# An investigation into the biological effects of indirect potable reuse water using zebrafish embryos



E. Lawton<sup>a,1</sup>, P. Antczak<sup>b,d,1</sup>, S. Walker<sup>a</sup>, E. Germain-Cripps<sup>c</sup>, F. Falciani<sup>b</sup>, E.J. Routledge<sup>a,\*</sup>

<sup>a</sup> Brunel University London, Institute for Environment Health and Societies, UB8 3PH, UK

<sup>b</sup> University of Liverpool, Institute of Systems, Molecular and Integrative Biology, L69 7ZB, UK

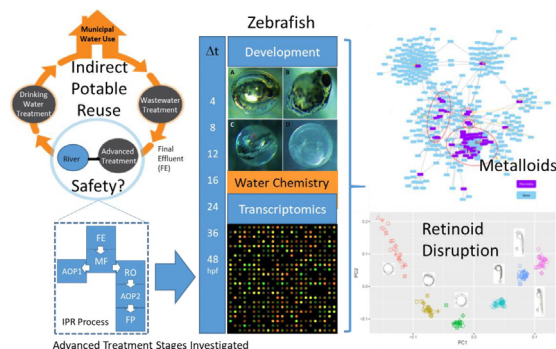
<sup>c</sup> Thames Water Utilities Limited, Reading RG1 8DB, UK

<sup>d</sup> University of Cologne, Faculty of Medicine and Cologne University Hospital, Center for Molecular Medicine Cologne, 50931 Cologne, Germany

## HIGHLIGHTS

- Safety of treatment technologies for indirect potable reuse investigated
- A low incidence of abnormalities seen in exposed embryos
- Transcriptomics linked teratogenicity to altered retinoid metabolism.
- Changes to retinoid metabolism were associated with metalloids.
- More research into retinoid disrupting chemicals needed

## GRAPHICAL ABSTRACT



## ARTICLE INFO

### Article history:

Received 1 March 2021

Received in revised form 18 May 2021

Accepted 19 May 2021

Available online 24 May 2021

Editor: Henner Hollert

### Keywords:

Advanced treatment

Water reuse

Toxicogenomics

Zebrafish

Biomonitoring

Retinoid

## ABSTRACT

Advanced treatment technologies are being assessed as a proactive measure to assist with the transformation of treated wastewater into a source of water for potable water production. We investigated the biological effects along an advanced water treatment pilot plant, using zebrafish embryos throughout early development. The study compared phenotypic observations with global transcriptome responses, enabling us to keep an open mind about the chemicals that might influence the biological activity. There was no evidence of acute toxicity at any treatment stage, but skeletal, cardiovascular and pigmentation changes occurred in a small proportion of embryos along the treatment process, and in a tap water; not detected in the aquarium water control. Reverse osmosis (RO) reduced the concentration of measured chemical contaminants in the water the most, while eliminating the occurrence of abnormalities detected in fish embryos. Conversely, advanced oxidation reversed the benefits of RO treatment by increasing the frequency of teratogenic and sub-lethal abnormalities seen. Using the molecular responses of zebrafish embryos to different IPR water, we report the bioactivity within the water at different stages of advanced treatment and associate these to perturbed biological functions. Transcriptomic analysis revealed alterations to the retinoid system, which was consistent with the observed teratogenic effects. Changes to tryptophan metabolism (associated with the production of melatonin required for the control of normal circadian rhythms) and somatolactin-beta (associated with normal pigmentation in fish) were also found. We show that underexplored forms of biological activity occur in treated wastewater effluent, and/or may be created depending on the type of advanced treatment process used. By integrating the available analytical chemistry we highlight chemical groups associated to this response. Our study shows that more detailed and in-depth characterisation of chemicals and biological pathways associated with advanced treatment water systems are needed to mitigate possible risks to downstream organisms.

© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

\* Corresponding author.

E-mail address: [edwin.routledge@brunel.ac.uk](mailto:edwin.routledge@brunel.ac.uk) (E.J. Routledge).

<sup>1</sup> First author.

## 1. Introduction

Global population growth is placing unprecedented demands on freshwater sources worldwide, raising concern about the long-term availability and quality of potable water sources (Brookes et al., 2014). The South East of England is already experiencing serious levels of water stress (EA, 2013). Under UK law, water companies are legally obliged to plan how they will manage the needs of future populations, deal with climate change (and water supply uncertainty) and develop – where needed – new water supply resources (Wood et al., 2006). Thames Water Utilities Limited (TW) is one of 12 UK private utility companies responsible for supplying potable water to 23% of the UK population and providing waste water treatment services to 27% of the UK population (*Building a Better Future*, 2019). As part of its water resource management plan process, TW constructed a pilot advanced water treatment plant to determine if indirect potable reuse (IPR) would be a viable approach in addressing the water security problems afflicting the South East of England. However, before implementing advanced treatment on a large scale, it is important to determine how much treatment is needed to strike the best balance between generating water that is safe to discharge into the environment and economic cost (National Research Council, 1998; Fatta-Kassinos et al., 2011; Dominguez-Chicas and Scrimshaw, 2010). Consequently, TW commissioned a programme of research to investigate the biological activity and possible health effects of exposure to treated water, taken from different stages of treatment within a pilot IPR water treatment plant.

Planned water reuse is not widely practiced in the UK. However, in the South East of England treated wastewater is an important component of river flow, especially in the summer months and periods of low water flow, where up to half of a river flow can be composed of treated wastewater effluent (Jobling et al., 1998). Consequently, unplanned reuse is typical, because treated wastewater from upstream towns and cities is abstracted from the downstream river flow in order to be processed back into drinking water. Advanced treatment via indirect potable reuse is a further intervention step in the current water reuse cycle. It aims to remove residual contaminants present in treated sewage effluent (normally released directly into rivers), using sophisticated physical and chemical processes before the final product water (FP) is blended back into an environmental buffer (rivers, surface reservoirs or aquifers). Advanced treatment is intended to make water reuse cycles quicker, protect the environment and ensure that downstream drinking water safety is upheld. In this pilot plant, the different stages of treatment start with relatively simple and low-cost processes like microfiltration (MF), and then progress onto more complex and costly processes like reverse osmosis (RO) and advanced oxidation (AOP).

The TW brief for this investigation was very broad - to investigate the safety (or possible health effects) of exposure to indirect potable reuse water at different stages of the advanced water treatment process. Our findings would help to determine how much treatment is necessary to strike the best balance between water safety and economic cost. Our challenge was to design an experiment that would highlight changes in biological activity of whole water samples at any stage along the IPR treatment process, associated with any chemical, at any concentration, or as part of a mixture effect. We also needed to be able to compare the biological effects along each of the different treatment stages, in order to evaluate their relative effectiveness.

Transcriptomic approaches in fish and mammals are gaining increasing traction as a way of evaluating the safety of municipal effluents and drinking water (Martinović-Weigelt et al., 2014; Vidal-Dorsch et al., 2013; Shi et al., 2014). The ability to quantify changes in the expression of many thousands of genes simultaneously in a single experiment enables toxicologists to identify important biological mechanisms and predict toxic outcomes caused by exposure to particular compounds and complex mixtures. Zebrafish continue to be a popular choice of model organism due to their small size, short generation time, high

fecundity, and ability to observe well-characterised early stages of development in optically transparent embryos ex-utero and the availability of established genomic tools and resources (Ruzicka et al., 2019). The zebrafish genome matches approximately 70% to human orthologs, supporting their use as a model species for biomedical research (Arend et al., 2017). For these reasons, zebrafish are increasingly used in toxicogenomic research (Williams et al., 2014), including water quality and environmental chemical exposure assessment (Caballero-Gallardo et al., 2016).

Toxicogenomic studies in fish illustrate the complex nature of chemical exposures on gene expression in specific tissues and at specific time points (typically following a short exposure time), corroborating findings from targeted studies (such as induction of VTG by estrogenic compounds) (Katsiadaki et al., 2010; Denslow et al., 1999). However, transcriptomic profiles and patterns are often complex to interpret in toxicological studies because chemical-induced biological events leading to adverse effects occur alongside general responses associated with cellular stress, apoptosis, and repair on whole genome responses. Consequently, clear pathways or modes of action, may not always be easily identified. The duration and timing of exposure, the tissue types that are analysed and the internal concentration of the chemical being tested are also likely to influence gene transcriptional profiles, thereby complicating further our understanding of the specific effects of any given compound. Moreover, few studies have tried to associate transcriptional changes with physiological effects, or generally, toxicological effects at a higher level of biological organisation (Fent and Sumpter, 2011), especially in exposures to complex environmental mixtures. However, toxicogenomic approaches have been effectively used to assess the toxicity of reconstituted water extracts taken from two municipal wastewaters and their receiving water using zebrafish larvae (Li et al., 2018).

Building on this knowledge, and to address this brief, we investigated the residual toxic biological effects of each treatment stage, by monitoring deviances away from normal development in zebrafish embryos exposed from fertilisation and throughout early development (Braunbeck and Lammer, 2006) – life stages that are particularly sensitive to disruption by chemicals, as explained by the ‘foetal origin of disease’ (Grandjean, 2008). Our strategy was to compare phenotypic observations with whole transcriptome responses in zebrafish throughout early development. Contrary to a single exposure time point (Li et al., 2018), our sampling approach extended throughout development, thereby improving our mechanistic understanding of the dynamic effects of treatment on key biological pathways related to health. Our approach differs substantially from targeted methods to assess the impacts of chemicals in water-treatment processes that employ a multiplicity of in vitro assays designed to capture different mechanistic endpoints (Escher et al., 2014; Dong et al., 2019) (e.g. nuclear receptor binding, enzyme inhibition, genotoxicity, cytotoxicity) and that enrich organic chemicals from the water using Solid Phase Extraction (SPE) thereby removing inorganics and altering the composition of the sample. Instead, we use whole water samples, without any form of additional treatment or extraction, so that both organic and inorganic phases are included. Although this study was not intended to identify chemical agents responsible for biological effects (e.g. using an Effects Directed Analysis approach (Desbrow et al., 1998)), we used available pilot plant water chemistry data obtained around the time that water samples were collected to determine whether observed transcriptomic responses could be explained by the measured organic and inorganic chemicals.

Our innovative approach enabled us to keep an open mind about which chemicals contained within the water at each sample stage might be influencing key biological pathways during early development. This resulted in both confirmatory data as well as identification of less obvious, and less studied, biological pathways associated with the residual effect, providing an improved understanding of real-world mixtures on biological systems during early development.

## 2. Methodology

### 2.1. Overall approach

A general summary of the overall methodological approach is shown in Fig. S1. We reared zebrafish embryos in various product waters sampled along the IPR pilot plant process, or aquarium water and a tap water as source of the aquarium water. Survival and developmental anomalies were recorded in each case at different stages throughout development. A detailed transcriptomic analysis was used to identify which genes were differentially expressed relative to the aquarium control water at each stage of development, and to identify key biological pathways altered by each treatment. By integrating perspectives gained from the transcriptional and developmental anomalies, we are able to propose mechanisms of action, and test these using toxicological approaches by exposing zebrafish embryos to chemicals detected in the treatment water and/or with a known mode of action.

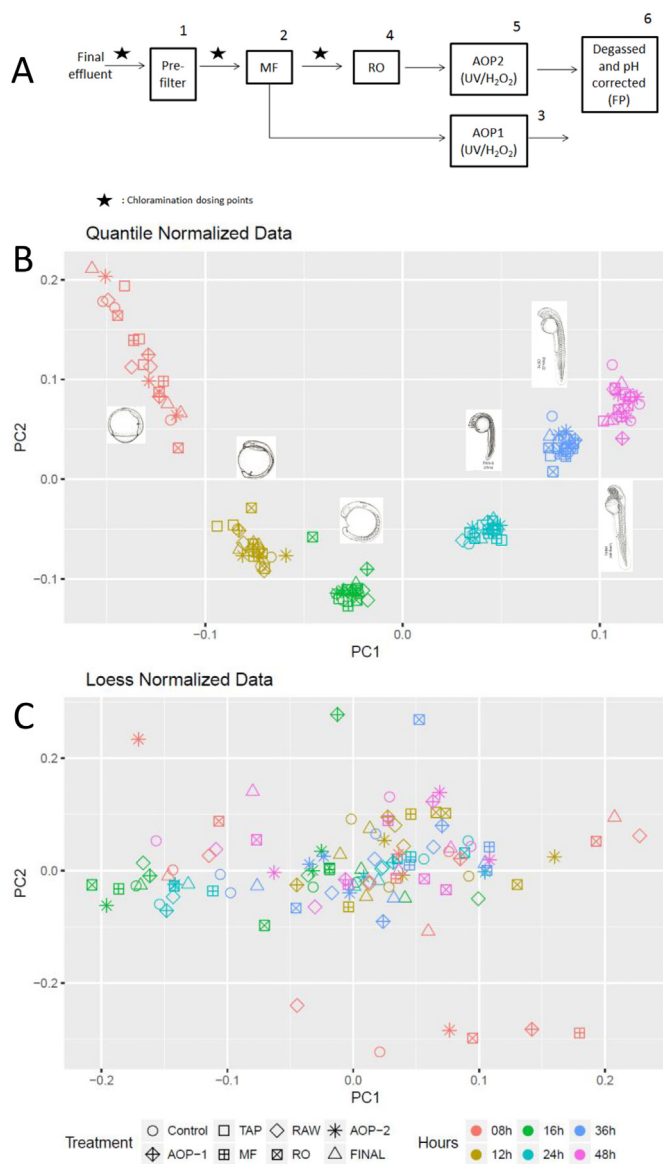
### 2.2. Pilot IPR facility

Thames Water Utilities' 600 m<sup>3</sup> d<sup>-1</sup> IPR pilot plant was commissioned in 2008 to generate data for any future plans to operate a full-scale project. The pilot IPR plant received final effluent from a conventional activated sludge plant processing domestic wastewater from a population of circa 870,000 at the time of study (personal communication Marie Raffin, Thames Water, UK).

The Sewage Treatment Works final effluent that feeds the IPR plant is pre-filtered using a 500 µm coarse filter, and then is filtered more finely through a microfiltration membrane (MF) process (Raffin et al., 2011). Chloramine is used to minimise biofouling in the pipework and equipment (Raffin et al., 2012), and can be added to the final effluent before and after pre-filtration, and after MF (Fig. 1A). After MF, one stream of MF permeate is treated with anti-scalant and sulphuric acid, before passing through a reverse osmosis (RO) membrane. It then goes through an Advanced Oxidation Process (AOP) where it is disinfected by ultraviolet light and dosed with hydrogen peroxide (AOP2). Finally, this stream (referred to as Final Product or 'FP') is corrected for pH and degassed. A second stream of MF permeate goes directly to a separate AOP system without reverse osmosis or anti-scalant/sulphuric acid (AOP1) and is the final product of this particular stream. The IPR plant was fully automated, with advanced water sampling instrumentation to monitor and control the process, and onsite supervisory control and data acquisition (SCADA) system to record trends and collect data on quality at various stages of the process for analysis in real time. As part of the continuous quality control of the IPR Plant TW generates chemical analytical data on a regular basis. We extracted the closest available date of available chemistry which coincided with data 24 h before completion of our 24 h composite sampling and thus represents the available chemicals in our samples very closely. Available water chemistry data has been summarised in Table S7. For privacy purposes, the specific lab and date of analysis have been removed from the table, and duplicated chemicals are measurements by multiple laboratories.

### 2.3. Water sample collection

We collected 24 h composite IPR plant's water samples using automated samplers set at 4 °C and stored these at 4 °C until needed (between 12 and 24 h after collection). General physicochemical properties of the aquarium control, tap water and IPR plant exposure waters are shown in Table S1. Water samples were sent to an approved laboratory by TW for routine chemical analysis, to identify compounds exceeding the Limit of Detection (LOD) (summarised in Fig. S4). Free chlorine in tap water was allowed to dissipate by letting the sample stand for 24 h before use.



**Fig. 1.** (A) Schematic of the IPR plant showing chloramine dosing points (.) and positions (1–6) where composite samples or grab samples were taken. (B) Principal component Analysis (PCA) plots of Quantile normalised data showing the large effect of developmental stage (4, 8, 12, 16, 24, 36 and 48 hpf) on gene expression in embryos exposed to different IPR product waters, tap and control. (C) Principal component Analysis (PCA) plot of cyclic loess normalisation PCA plot of data normalised against aquarium water to reveal the effects of the IPR treatment alone. In each case, each point represents the integrated expression of 15,000 genes.

### 2.4. Zebrafish culturing and spawning

We sourced adult zebrafish (*Danio rerio*) (Tübingen strain) from University College London (UCL, UK). The fish were separated according to sex and placed into 10 l flow-through tanks receiving dechlorinated carbon-filtered (5 and 10 µm) tap water (aquarium header water) fed from header tanks at a flow rate of 20 l/h at 26–28 °C with a photoperiod of 14 h Light: 10 h Dark. Fish were fed 3 times a day; twice with flake food (King British Tropical flake food, Lillicos, Surrey) and once with adult brine shrimp (Tropical Marine Centre, Gamma irradiated). All tubing (flow-line, air-lines, siphon, and the attached grids) are made of medical grade silicon (VWR, UK).

To induce spawning, we placed sexually mature male and female adult zebrafish together overnight in 9.5 litre breeding tanks at a ratio of 2 males to 4 females. Each breeding tank was fitted with a metal grid allowing the eggs to fall to the bottom of the tank. We selected

and collected healthy fertilised eggs by eye the following morning (~1 h after dawn) and placed the embryos (aged between 1 and 2 hour post-fertilisation) into 300 ml crystallising dishes containing aquarium water. All procedures were performed in compliance with the Animals (Scientific Procedures) Act 1986 and institutional guidelines following appropriate institutional committee approval. The research was conducted with 'Animal Research: Reporting In Vivo Experiments' (ARRIVE) guidelines (Kilkenny et al., 2013) in mind.

## 2.5. Temporal effects of IPR water exposure on development

In order to investigate the phenotypic effect of exposure we took various physicochemical readings including dissolved oxygen (HACH oxygen and temperature probe), pH, general hardness, carbonate, nitrate, and nitrite (API 5 in 1 test strips), and ammonia (API ammonia test strips) in water samples at the start of every exposure. We warmed a 24-well plate containing 2 ml of an IPR product water/well (or aquarium header water control or tap water) to 28 °C in an incubator while newly fertilised embryos were collected and sorted. We placed one embryo into each well and took temperature readings inside the incubator at the start of the exposure with a thermometer, as well as continuous readings throughout the experiment using two Tiny Tag data loggers.

We observed embryos at time intervals (4, 8, 12, 16, 24, 36, and 48 hour post-fertilisation (hpf)) using a Leica MZ FL III fluorescence stereomicroscope and the Leica DC 300F digital image recording system. Deviances away from normal developmental expectations at each time point were recorded. The experiment was repeated three times over a period of 3 months. The endpoints we used to categorise embryos as 'normal' or 'abnormal' came from a number of sources (Table S2), including the OECD background document for the fish embryo toxicity tests (Braunbeck and Lammer, 2006; Nagel, 2002; Schulte and Nagel, 1994; Braunbeck and Lammer, 2005). Differences in the frequency and severity type of abnormalities (namely, Severe - curvature of the spine/twisted spine; malformed head; enlarged heart; no tail; stunted tail; no otolith development; gap in yolk sac. Sublethal - no eye development; enlarged yolk sac; blood clot in the heart; reduced body pigmentation; reduced eye pigmentation; misshapen eyes. Lethal - no somites; no head) in the total population of zebrafish embryos exposed to the different waters were compared using the Diversity Permutation Test in PAST v3.17 software (Hammer et al., 1999) using the Simpson Biodiversity Index. This approach enabled us to compare the diversity of abnormalities (both frequency and the number of different types) found in each treatment group. At 48 h, we transferred the exposed groups of embryos into RNAlater® stabilisation solution (Life Technologies, UK) at 4 °C for 24 h and then stored them at -80 °C.

## 2.6. IPR water exposures for gene expression analysis

### 2.6.1. Embryo exposures and sample preparation

To characterise the transcriptomic profiles we randomly allocated 50 embryos to 200 ml of IPR water (and aquarium and tap waters) in 300 ml glass crystallising dishes and used a glass petri dish as a lid. Dishes were incubated at 28 °C in large fish tanks receiving a flow of heated water from the header tanks. Every IPR product water (FE, MF, AOP1, RO, AOP2, FP) and reference water samples (Brunel University London aquarium and tap water) consisted of six independent sampling time-points (at 8, 12, 16, 24, 36 and 48 hour post fertilisation), carried out in duplicate, resulting in 96 dishes for each experiment. We sampled embryos throughout development to capture transient biological effects occurring during embryogenesis of possible relevance to the abnormalities seen.

At each time point, we photographed one 'normal' embryo from each dish and counted and removed any dead embryos. Abnormal embryos were photographed and preserved individually in RNAlater® (0.6 ml/embryo) in case they were needed at a later date. Finally, the remaining embryos were pooled and preserved together in 1.4 ml of

RNAlater®. It was decided not to include RNA from phenotypically abnormal embryos in the microarrays as they may have a disproportionate effect on the pooled sample. We carried out three independent exposures over a period of approximately one month.

### 2.6.2. RNA extraction and hybridization

We extracted RNA using the RNeasy Mini Kit (QIAGEN, USA) following the manufacturer's protocol for the preparation of RNA from tissue (QIAGEN, 2012) with modifications for use with fish embryos. Briefly, embryos were removed from the -80 °C freezer and defrosted on ice, after which we removed the RNAlater® using a pipette. We then homogenised the embryos using 350 µl Buffer RLT (containing 10 µl β-mercaptoethanol/ml of buffer) by repeatedly drawing up and dispensing the mix using a 1 ml syringe. To prevent the filter from blocking, we passed the resulting lysate 10 times through a blunt 20-gauge needle (0.9 mm diameter) and used "On-Column DNase Digestion with the RNase-Free DNase Set" to recover the RNA. Finally, we eluted the RNA from the column with 36 µl RNase-free water. We quantified RNA yield using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer and visualised the RNA purity and quality by electrophoresis (1.5% agarose gel with ethidium bromide). We used a One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labelling kit supplied by Agilent Technologies, with a final concentration of 75 ng/µl total RNA. The procedure included the following steps; cDNA synthesis, cRNA synthesis and amplification, cRNA purification, preparation of hybridization sample, 17 hour hybridization (65 °C), wash, scan, and feature extraction (Agilent, 2014). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar, 2002) and are accessible through GEO Series accession number GSE152131 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152131>).

### 2.6.3. Pre-processing and statistical analyses

We normalised and analysed our data using the statistical programming environment R (R Core Team, 2016). The two different normalisation approaches were: 1) quantile normalisation ('normalize.quantiles' function from the preprocessCore package) and 2) cyclic loess normalisation against their respective experimental and developmental stage control ('normalizeWithinArrays' function in the limma package). The expression level of each gene, within each treatment group and time point, are normalised against their corresponding aquarium water control group gene. We did not normalise gene expression along the IPR treatment to tap water, because chemicals present in IPR samples may occur in tap water, thereby potentially masking residual transcriptomic effects along the advanced treatment process. Loess normalisation takes into account the effect of development on the molecular response, and in doing so will emphasize the effect of treatment. We used principal component analysis (PCA) to reduce the overall dimensionality of data and for visualisation purposes. We analysed differential gene expression using the Significance Analysis for Microarrays (SAM) methodology as implemented in the samr package (Tusher et al., 2001). To identify individual genes that are differentially expressed at each developmental time point along the treatment process (expressed as log-fold changes), we carried out a two-group comparison in limma on each individual gene expression dataset against their corresponding control (e.g. 8 h Aquarium Water against 8 h Final Product).

### 2.6.4. Time-course and pseudo-time-course analysis

To help identify genes whose relative expression gradually increased or decreased during development (8 h–48 hpf) in each of the IPR treatments, we used a time-course analysis in the samr package (Tusher et al., 2001). For the pseudo-time-course analysis we assumed that a relationship between the different treatments is present. We only focused on the following direction of flow 'Pre-MF-RO-AOP2-FP' and repeated the analysis for each timepoint. This enabled us to identify genes that change across the direction of treatment for each timepoint, to highlight genes responding gradually to change along the treatment process.

### 2.6.5. ANOVA

Unlike the time-course type analysis, an ANOVA will identify any significant change at any treatment position or time-point. To aid in biological interpretation we used pathway indices as an input to a 2-factor ANOVA on both the quantile and loess normalised data. We only considered pathways for which more than 5 genes were present for further analysis:

$$\text{pathway} = \text{stage}_{\text{dev}} + \text{treatment} + \text{stage}_{\text{dev}} * \text{treatment} + \text{experiment}$$

where 'stage<sub>dev</sub>' represents the developmental stage of the fish (hpf), 'treatment' the stage in the IPR plant (i.e. positions 1–6 in Fig. 1A) and 'experiment' the day on which the experiment was performed. We adjusted the *p*-values using a False Discovery Rate (FDR) correction (Benjamini and Hochberg, 1995) using the *p.adjust* function in R.

### 2.6.6. Linking gene expression changes across IPR stages

To identify whether there is a relationship between the available chemistry and gene expression profiles that we developed, Pearson correlation was used. First chemical concentrations were transformed with log<sub>10</sub> and then combined with each individual gene within our dataset. For those comparisons where sparsity resulted in <6 overlapping samples, and where the variation within the chemical concentrations was below 0.1 were removed to ensure that only relevant connections were identified. Resulting list was filtered for correlations with at least an |*r*| > 0.75. The resulting connections were then represented as a graph and visualised using Cytoscape.

### 2.7. Developmental exposures to known chemicals

Following detailed evaluation of the responses of zebrafish embryos to IPR treatment waters, we exposed zebrafish embryos up to 48 hpf to specific chemicals based on (i) their presence in treatment waters and/or (ii) reported mode of action, in order to corroborate the genomic and phenotypic effects we observed based on the literature (Fig. S1). Propiconazole (an antifungal), ibuprofen (NSAID) and ER50891 (retinoic acid receptor alpha antagonist) were chosen for these tests, and the rationale these choices is explained further in the Results section. All chemicals were analytical grade standards and were tested using three doses based on environmental relevance (if known). We

prepared a master stock of 4-[5-[8-(1-Methylethyl)-4-phenyl-2-quinolinyl]-1H-pyrrolo-2-benzoic acid (ER50891 - 250 mg/l; Tocris, UK - ≥99% purity) by dissolving 2.5 mg of chemical in 10 ml analytical grade DMSO, and made working stocks of 20, 10 and 5 mg/l ER50891 by diluting the master stock further with DMSO. We aliquoted 20 µl of each working stock (or DMSO solvent control) into acid and solvent rinsed Pyrex dishes in triplicate and added 200 ml of aquarium water to produce final ER50891 nominal concentrations of 2 µg/l, 1 µg/l and 0.5 µg/l (0.01% DMSO). The same approach was used to expose developing zebrafish embryos to Propiconazole (≥99% purity; Sigma-Aldrich, UK; nominal doses 0, 0.1, 1.0 and 10 mg/l), and Ibuprofen (≥99%; Sigma-Aldrich, UK; nominal doses of 0, 4.0, 40 and 400 µg/l). We added 50 fertilised embryos (between 1 and 2 hpf) to each dish using a Pasteur pipette, and placed them into an incubator set at 28 °C for 48 h. Up to three independent exposures were carried out with embryo observation as described in Section 2.6.1.

## 3. Results

### 3.1. Water physicochemistry across IPR stages

Ammonia (0–3 mg/l), nitrate (40–80 mg/l), nitrite (0–3 mg/l) and hardness (180–240 mg/l) were highest in the early stages of treatment (Final Effluent to AOP1) with pH levels of 7.5–8.0. Water samples taken after RO treatment had decreased pH (6.0–7.0), hardness (0–40 mg/l), ammonia (0–1 mg/l), and nitrite and nitrates were non-detectable. The RO and Final product were similar in all measurements taken. Tap water and aquarium water had pH and hardness levels similar to final effluent, and ammonia, nitrate, and nitrite levels similar to the RO and Final Product waters (see Table S1).

### 3.2. Characterisation of abnormalities in zebrafish embryos exposed to IPR water

The mean frequency of spontaneous abnormalities in all exposed embryos at 48 hpf was typically low, ranging from 0% (control and RO), 2.3% and 2% in tap water and final effluent, respectively, and reaching 3.7% in the AOP2 treatment. We observed differences in abnormality types and severity class (Teratogenic, Lethal and Sub-Lethal) during advanced treatment, and in the tap water (Table 1), without

**Table 1**

Total percentage of different abnormalities detected in embryos at 48 hpf exposed to aquarium control water (AqC), Brunel tap water (TAP), and treatment water taken from the IPR pilot plant (Final Effluent (FE); Microfiltration (MF); Advanced Oxidation (AOP1); Reverse Osmosis (RO); Advanced Oxidation with RO (AOP2) and Final Product Water (FP). Abnormalities were recorded from 6 independent experiments using two different approaches (individual embryos placed in 24-well plates or groups of 50 embryos in petri dishes). Data was collected over a period of four months. Number of abnormal embryos and the total number analysed: FE 4/199; MF 2/246; AOP1 4/252; RO 0/258; AOP2 7/188; FP 1/182; TAP 6/263 and AqC 0/248. Differences in total number of embryos are due to a combination of early mortality, technical difficulties in sample collection, and/or the availability of embryos. Classification of abnormalities is based on Braunbeck and Lammer (2005), Nagel (2002) and Schulte and Nagel (1994). CS: curvature of the spine/twisted spine; MH: malformed head; EH: enlarged heart; NT: no tail; NO: no otolith development; NE: no eye development; EY: enlarged yolk sac; BCH: blood clot in the heart; NP: no pigmentation; NC: no circulation; NM: no movement; NS: no somites; NHB: no heartbeat; UT: undetached tail. Abnormality type; Teratogenic (T), Sub-Lethal (SL) and Lethal (L).

Abnormality	Severity	IPR Process Waters						Reference	
		FE	MF	AOP1	RO	AOP2	FP	TAP	AqC
CS	T	0.00	0.41	0.40	0.00	1.60	0.00	1.14	0.00
MH	T	0.00	0.00	0.00	0.00	0.00	0.00	0.38	0.00
EH	T	0.00	0.00	0.40	0.00	0.53	0.00	0.00	0.00
NT	T	1.01	0.00	0.40	0.00	0.00	0.55	0.00	0.00
NO	T	1.01	0.00	0.00	0.00	0.00	0.00	0.38	0.00
NE	SL	1.01	0.00	0.00	0.00	0.00	0.00	0.38	0.00
EY	SL	0.00	0.00	1.19	0.00	1.06	0.00	0.00	0.00
BCH	SL	0.00	0.00	0.40	0.00	1.06	0.00	1.14	0.00
NP	SL	2.01	0.41	0.00	0.00	0.53	0.00	0.00	0.00
NC	SL	1.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NM	SL	1.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NS	L	1.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NHB	L	1.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
UT	L	1.01	0.00	0.00	0.00	0.00	0.00	0.38	0.00

treatment-related delays in developmental rate. There were nine abnormality types observed in the embryos exposed to FE, MF had 2 of these, AOP1 and AOP2 each had five, RO had none, FP had one (teratogenic) and tap water had six. Like RO, no abnormalities were seen in the Aquarium water. The abnormalities could be broadly classified into skeletal anomalies (curvature of the spine, absence of otolith development or absence of a tail – see Fig. S2B), cardiovascular anomalies (e.g. enlarged heart, pericardial oedema, and blood clot in heart – see Fig. S2C), and pigmentation effects (e.g. an absence of pigment – see Fig. S2D). Significant differences in the diversity (frequency and type) of abnormalities occurred between different treatment groups (Table 2); most notably the source water (Final Effluent) had significantly more abnormalities compared to all other waters (except Tap), and the aquarium control water had significantly fewer abnormalities compared to the other waters (excluding MF, RO, and FP).

### 3.3. IPR exposure affects key drug metabolism components and somatolactin-β

To understand the effect treatment and developmental stage had on our zebrafish we initially visualised the samples using PCA plots. Embryo development had the most profound effect on the molecular responses relative to any other variable, including exposure to IPR water treatment, replicates, and experiment number (Fig. 1B). To resolve the effect of exposure to the different water treatment stages, we loess normalised each of the treatments against their respective control samples. This accounts for possible uncontrolled variables during the experimental period, and thereby reveals differences in gene expression caused by exposure to the different IPR product waters (Fig. 1C).

For each developmental stage and IPR treatment process we identified individual genes that were up and down regulated relative to the aquarium control (Table 3). Somatolactin-β was up-regulated at 48 hpf in the embryos exposed to tap water, FP, AOP2 and RO product waters. Somatolactin-β gene transcription remained unchanged at all other time points and in the embryos exposed to the other product waters. Cytochrome P450, family 1, subfamily A was also up-regulated in embryos exposed to early treatment stages (FE, MR and AOP1), and at various developmental time points (Table 3). Cytochrome P450, family 1, subfamily B was up-regulated in all the same treatments, but only at 12 hpf.

In order to establish (i) whether differences occurred in global gene expression between treatments, and (ii) which treatment stages were most dissimilar with other treatment stages, the smallest FDR value associated with statistical significance between treatments was selected. In this analysis, the lower the FDR the more likely a statistical difference between the molecular responses is observed, and that this is true. While this does not include the number of genes, it does include the highest observed statistical difference, and can act as a guide of dissimilarity across the treatments. Fig. 2A illustrates the most significant differences in global gene expression, indicated by the red arrows at the top of the flow chart and the FDR value beside the arrow; the green

**Table 2**

Differences in the frequency of different abnormalities in zebrafish embryos exposed to treated final effluent (FE), advanced treatment water (MF, AOP1, RO, AOP2, FP), a tap water and associated aquarium water using the Diversity Permutation Test in PAST software and the Simpson Index as a measure of differences in the diversity of abnormalities in each treatment group. Red boxes  $P < 0.05$  and blue boxes  $P < 0.1$ .

	MF	AOP1	RO	AOP2	FP	Tap	AqC
FE	0.0001	0.0023	0.0001	0.003	0.002	0.123	0.0001
MF	X	0.1589	0.2371	0.6552	1	0.0309	0.5013
AOP1		X	0.0164	0.343	0.1166	0.5616	0.0149
RO			X	0.0753	0.6207	0.0017	1
AOP2				X	0.6327	0.1622	0.0813
FP					X	0.0556	0.4326
Tap						X	0.0023
AqC							X

**Table 3**

Comparison of expression of differentially regulated genes identified in embryos exposed to tap water and product waters from the IPR treatment process at different developmental stages (hours post fertilisation; hpf) compared to their respective aquarium water control. Analysis carried out using a two-group comparison in Limma using the 'topTable' function on each individual gene expression dataset against their corresponding control. Only time points where one or more genes were differentially expressed using this analysis are shown. ↑ denotes up-regulation and ↔ denotes no change in regulation. For more information (including log-fold change) see Table S3.

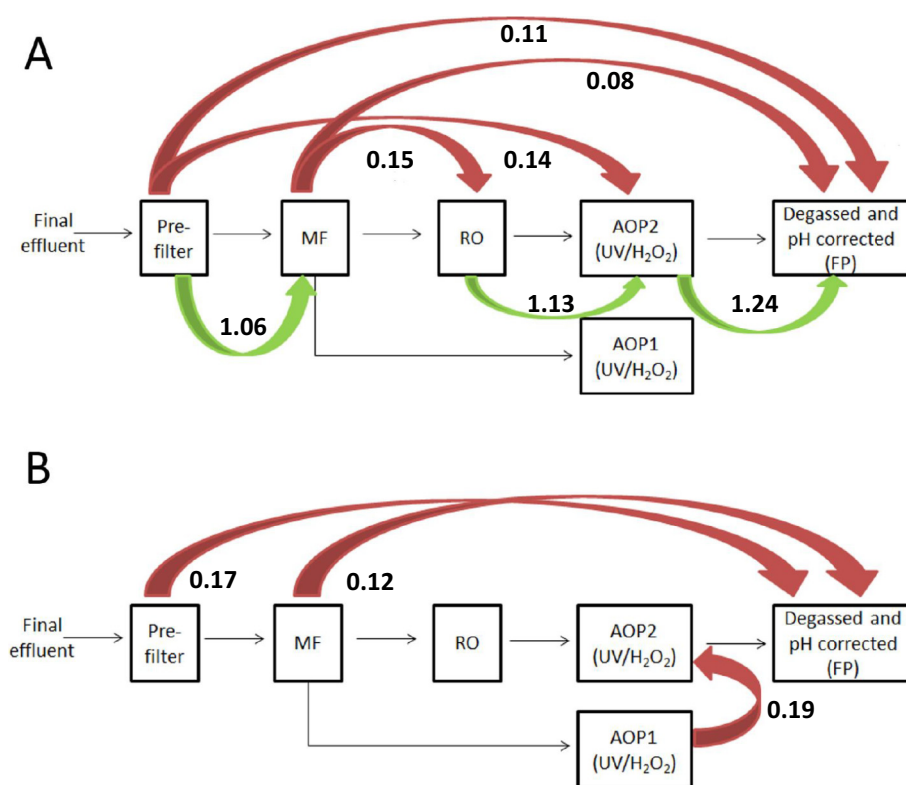
Treatment	hpf	Cytochrome P450, family 1, subfamily A	Cytochrome P450, family 1, subfamily B, Polypeptide	Somatolactin-β
FE	12	↑	↑	↔
	24	↑	↔	↔
	48	↑	↔	↔
MF	12	↑	↑	↔
	24	↑	↔	↔
	36	↑	↔	↔
	48	↑	↔	↔
AOP1	12	↔	↑	↔
	24	↑	↔	↔
	48	↑	↔	↔
RO	48	↔	↔	↑
AOP2	48	↔	↔	↑
FP	48	↔	↔	↑
Tap	48	↔	↔	↑

arrows underneath the flow chart show where the differences in gene transcriptional responses between treatments were much less pronounced. In general, the smallest FDRs occurred between treatments that were further apart along the IPR process, for example final effluent (following a pre-filtration step) and final product (FDR of 0.11). However, microfiltration and reverse osmosis are “next” to each other in the IPR treatment process and have a much lower FDR (0.15), indicating that the statistical differences in gene expression between these treatments are more likely to be true. Certain treatment processes (such as microfiltration of pre-filtered final effluent) appeared to show no measurable effects on global gene responses compared to embryos exposed to final effluent (FDR 1.06). In contrast, there was a strong statistical difference between post-MF and the final product water (FDR 0.08). Therefore, global gene responses to pre-filtered final effluent and microfiltration are much more similar to one another compared to treatments further along the process. No substantial differences were observed in global gene expression between reverse osmosis and AOP2 (FDR 1.13) and AOP2 and final product (FDR 1.24).

### 3.4. Pathway analysis identifies additional developmental and metabolic pathways

To better characterise the exposure system, we opted to analyse the response on the level of pathway indices. Here instead of using single gene expression we summarise the activity of a pathway of multiple genes into a single variable and used that as an input to an ANOVA. Using this approach, we assessed the influence of each experiment (1, 2 or 3), developmental stage and IPR treatment stage on pathway indices. Quantile and loess normalised data revealed 329 and 269 pathway indices to be differentially expressed based on experiment, and 392 and 289 pathways indices to be differentially expressed due to developmental stage, respectively. In contrast, the number of pathway indices that were differentially expressed in repeat experiments based on IPR treatment process (sample) was only 10 and 5 using the quantile and loess normalised data, respectively. These pathways were all broadly involved in hormone synthesis/metabolism and detoxification (see Table 4).

Fig. 2B shows differences in gene pathway responses between different treatments at different stages of development using the FDR as a measure of similarity and reliability. Only three treatment combinations



**Fig. 2.** A. Illustration of the IPR treatment plant with the selection of the minimum FDRs for loess normalised dataset using SAM time-course analysis at gene level using the signed approach. The lower FDRs indicate larger difference in global gene expression between treatment stages. The complete list of minimum FDRs are displayed in Table S4. B. Illustration of the IPR treatment plant with the selection of the minimum FDRs for quantile normalised dataset using SAM time-course analysis at pathway level using both the signed and slope approach. The FDRs shown on the figure are significantly different. The complete list of minimum FDRs are shown in Table S5.

produced significant differences in the expression of gene pathways using this analysis approach. These included differences between the pre-filtered final effluent and the final product water (FDR 0.17), and between the microfiltration and final product water (FDR 0.12). A significant difference in gene pathway responses were also observed between the two AOP treatments (FDR 0.19), where the product water from the AOP2 process had previously undergone microfiltration and the addition of anti-scalant and sulphuric acid prior to reverse osmosis, whereas the product water from the AOP1 treatment had undergone microfiltration alone. FDR values were typically higher in all other comparisons, which indicates that there is a higher proportion of type 1 errors, and therefore a higher probability of recording a false positive in these comparisons.

We used a pseudo time-course to identify differentially expressed pathways along the entire treatment process at each developmental time point using the quantile-normalised dataset. This approach identifies genes that change across the direction of treatment for each timepoint, to then assemble pathways that are responding gradually to change along the treatment process. The time-course followed the treatment order FE → MF → AOP1 → RO → AOP2 → FP → Control.

**Table 4**

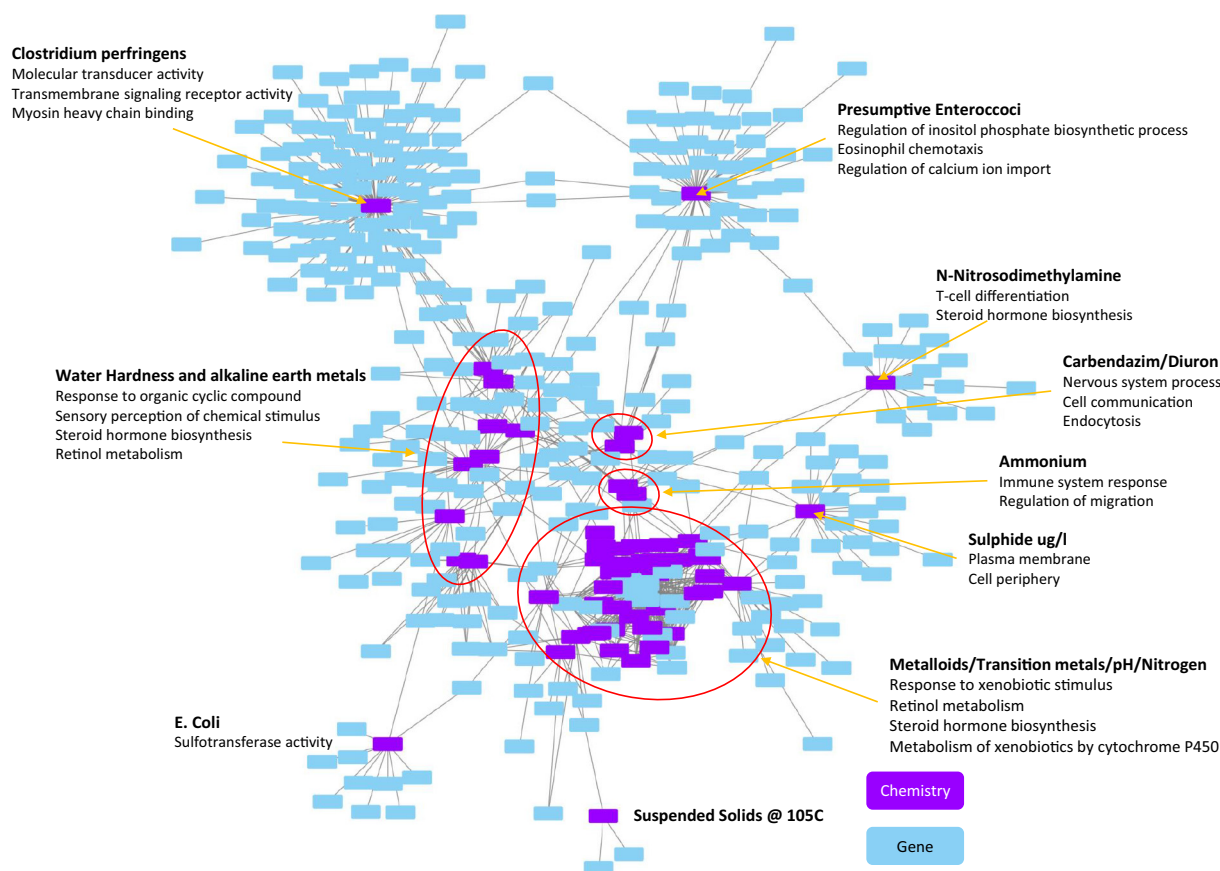
2-Factor ANOVA Pathway level. The '+' means that the corresponding pathway was differentially expressed in the embryos from experiment (Exp) 1, 2 or 3 using the two different methods of analysis.

Pathways	Quantile normalised data method			Loess normalised data method		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
Steroid hormone biosynthesis	+	+	+	+		
Tryptophan metabolism	+			+		
Retinol metabolism	+	+		+		
Metabolism of xenobiotics by cytochrome P450	+	+		+	+	
Drug metabolism-cytochrome P450	+					
Drug metabolism-other enzymes	+					

Melanogenesis pathways were down-regulated at 12 h whereas the Hedgehog signalling pathway was upregulated at 36 h. Tryptophan metabolism (used in the synthesis of brain neurotransmitters melatonin – modulator of circadian rhythms – and serotonin) was also altered at different stages of development along the treatment process. The most commonly altered pathways affected by treatment during development were changes to purine and pyrimidine pathways associated with maintenance of nucleotide levels in tissues (Table S6).

### 3.5. Analytical chemistry associated to biological responses

In order to test whether the observed responses could be explained by the associated chemistry, we retrieved the routine chemical concentrations, developed by TW during the sampling period, and correlated these to the observed gene expression. Out of the 178 analytical measurements undertaken, as part of the chemical analysis, we focused on 57 with sufficient variance and availability to correlate with gene expression profiles. Chemicals that did not change between the three sampling dates, or chemicals for which many of the datapoints were below the level of detection were removed. Fig. 3 visualises the outcome of this



**Fig. 3.** Correlation network representing the relationship between chemical analytical features and gene expression profiles. Nodes marked in purple represent the chemistry and nodes in blue genes. Closely positioned nodes are highly correlated with each other. To identify the functional enrichment of genes associated to the analytical chemistry the first neighbours of closely positioned analytical chemistry nodes were performed. The resulting pathways are highlighted underneath the node group description shown. For further detailed annotation of chemical analytical features and gene expression profiles please see Fig. S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

approach and highlights several overlaps with key functional terms. Interestingly, *N*-Nitrosodimethylamine was associated with T-cell differentiation and steroid hormone biosynthesis relevant genes which has long been a known landmark of NDMA exposure (Desjardins et al., 1992). Carbendazim and Diuron, which are a fungicide and algicide respectively, were closely related to cell communication, nervous system processes, and endocytosis functions; all hallmarks of this subtype of chemicals. A closer look across much of the responses identified in this analysis highlighted drug and retinol metabolism linked to metal and metalloids responses as well as water characteristics (Fig. 3).

### 3.6. Exposure to a retinoic acid receptor alpha antagonist (ER50891) replicates abnormalities

Our analysis identified drug metabolism-relevant pathways associated to the different exposures across the IPR treatment plant. Moreover, our analysis (Fig. 3) highlighted the potential genes and functions that directly link to water quality parameters measured, and showed that the previously identified drug metabolism pathways were associated with metalloids and transition metals. In the same group we also identified steroid hormone biosynthesis and the retinol metabolism pathways. The variety and type of abnormalities seen (skeletal, craniofacial, cardiovascular and pigmentation) indicated disruption to critical pathways in early morphogenesis, and therefore the retinoid system was selected for further investigation.

To test the hypothesis that disruption to the retinoid system was a key mechanism responsible for the abnormalities observed in embryos

reared in the IPR process water, we exposed developing zebrafish embryos to ER50891 (a retinoic acid receptor alpha antagonist), Propiconazole (a triazole fungicide), and Ibuprofen (a non-steroidal anti-inflammatory drug; NSAID). Propiconazole was chosen because it is reported to reduce hepatic retinoid levels in mice (Chen et al., 2009) by disrupting Cyp26-induced inactivation of endogenous retinoic acid in tissues with associated skeletal effects (Novák et al., 2008; Menegola et al., 2006) and because azole fungicides were detected in the raw water input of the IPR pilot plant (e.g. Clomitrazone - Table S7). Ibuprofen was tested because non-steroidal anti-inflammatory drugs (NSAIDs) are widely detected in the environment (Corcoran et al., 2010), have known cardiotoxicity associated with COX-2 inhibition (Jane Mitchell et al., 2019; Zhang et al., 2020) and was measured in the raw water input (see Table S7). Although not measured in the IPR water, ER50891 was chosen because it is known to inhibit effects of endogenous retinoic acid by binding to the retinoic acid receptor (Kikuchi et al., 2001). Developmental exposure to Propiconazole and Ibuprofen did not cause clear dose-dependent increases in abnormalities seen in the IPR and tap waters (see Table S8), although there was evidence of pigmentation reduction in a small proportion of embryos exposed to propiconazole and cardiotoxicity with Ibuprofen at the highest dose. In contrast ER50891 caused a dose-dependent increase in the frequency of abnormal embryos from 3.4% in 500 ng ER50891/l to 12.6% in 1 µg/l (Table 5). Furthermore, multiple abnormalities were observed in the embryos exposed to ER50891 that were also seen in the embryos exposed to AOP1, AOP2 and tap water, including skeletal malformations (curved spine, no tail, malformed



**Table 5**

Frequency of abnormalities detected in zebrafish embryos exposed to nominal concentrations (500 ng/L and 1 mg/L) of a selective retinoic acid receptor (RAR $\alpha$ ) antagonist ER50891 and the solvent control (SC: DMSO, 0.01% final concentration) after 24 h and 48 h post fertilisation. Number of abnormal embryos and total number analysed at 48 h: SC 3/146; 500 ng/L 11/324; 1000 ng/L 17/134. Classification of abnormalities was based on Braunbeck and Lammer (2005), Nagel (2002) and Schulte and Nagel (1994). Teratogenic (T) - CS: curvature of the spine/twisted spine; MH: malformed head; EH: enlarged heart; NT: no tail; ST: stunted tail; NO: no otolith development; GYS: gap in yolk sac. Sublethal (SL) - NE: no eye development; EY: enlarged yolk sac; BCH: blood clot in the heart; RBP: reduced body pigmentation; RPE: reduced eye pigmentation; ME: misshapen eyes. Lethal (L) - NS: no somites; NH: no head.

Abnormality	Severity	SC		500 ng/l ER50891		1000 ng/l ER50891		2000 ng/l ER50891	
		24 hpf	48 hpf	24 hpf	48 hpf	24 hpf	48 hpf	24 hpf	48 hpf
CS	T	0.68	1.37	1.49	3.73	4.48	2.99	4.05	1.35
MH	T	0.68	0.00	5.22	2.24	8.21	0.75	4.05	0.68
EH	T	0.00	1.37	0.75	4.48	9.70	9.70	8.78	4.05
NT	T	0.00	0.00	7.46	1.49	6.72	3.73	3.38	2.03
ST	T	0.00	0.68	0.75	0.75	9.70	2.24	1.35	0.68
NO	T	0.00	0.00	7.46	0.00	6.72	2.99	1.35	1.35
GYS	T	0.68	0.00	3.73	1.49	10.45	3.73	4.73	0.00
NE	SL	0.68	0.00	4.48	0.00	5.22	0.75	1.35	0.00
EY	SL	0.00	0.68	4.48	0.75	8.21	0.75	0.68	0.00
BCH	SL	0.00	0.68	0.00	1.49	0.00	0.75	0.00	1.35
RBP	SL	0.00	0.00	2.99	0.00	0.75	0.00	0.00	0.00
RPE	SL	0.00	0.00	0.75	0.00	3.73	0.00	0.00	0.00
ME	SL	0.00	0.00	0.00	0.00	0.75	0.00	0.00	0.00
NS	L	0.00	0.00	7.46	0.75	7.46	3.73	2.70	2.03
NH	L	0.00	0.00	5.97	0.00	2.99	1.49	0.68	0.00

head, no otolith), cardiovascular effects (enlarged heart, blood clot in heart), and reduced body and eye pigmentation.

#### 4. Discussion

TW's broad research question inspired us to take the ambitious step of departing from traditional hypothesis-led approaches and to investigate the IPR treatment process with no pre-conceptions in favour of particular chemicals or specific biological effects. Our aim was to determine if phenotypic abnormalities could be related to specific transcriptomic pathways associated with toxicity, at different stages of embryonic development, in zebrafish exposed to whole water samples taken along the entire IPR process. Such an approach has not been attempted before.

##### 4.1. Zebrafish survival and abnormalities throughout the IPR process

The zebrafish embryos survived equally well in water taken from all treatment stages, indicating that no acute toxicity occurred to the developing embryos along the entire treatment process. Nevertheless, a small number of distinctive spontaneous abnormalities (~1–2% frequency) occurred in the IPR treatment groups with the exception of RO where no abnormalities were seen. These included teratogenic abnormalities, such as altered pigmentation, cardiovascular anomalies and skeletal deformities that were not observed in the embryos exposed to aquarium control water. Their occurrence and association to the IPR process water was only apparent because of the large number of fish embryos used in our studies. Often, in IPR process waters, multiple abnormalities occurred together within a single embryo which is why the total number of abnormalities detected is greater than the total number of abnormal embryos.

Interestingly, exposure of the embryos to a tap water (introduced for a comparison with the final product waters; AOP1 and FP) produced a low incidence of the same types of abnormalities seen in embryos exposed to the IPR process waters, and in particular AOP1 and AOP2. As the tap water was supplied by a different water utility company, it would have been more appropriate to use tap water from the same source (being the end-of-pipe product from unplanned reuse from the same catchment area). Notwithstanding, as the FP water from indirect potable reuse is not intended for direct municipal supply, the tap water comparison has little relevance with respect to current reuse

scenarios. Moreover, the significance (if any) of these findings to human health would need to be the subject of a much more thorough investigation.

##### 4.2. Relationship of IPR water physicochemistry to the observed effects

It is conceivable that changes to the physicochemical properties of the water (e.g. pH, nitrite) along the IPR process (Table S1) may have caused the observed teratogenic abnormalities. However, zebrafish embryos tolerate a wide pH range (between 6.0 and 8.5) and wild populations occur naturally in waters with pH between 6.6 and 8.2 (McClure et al., 2006). We found no evidence of pH-induced teratogenicity in the literature; zebrafish can survive well in acidic water as low as pH 4.0 under laboratory conditions (Kwong et al., 2014). In addition, the nitrite levels measured along the IPR treatment are not likely to cause teratogenic effects in zebrafish during embryogenesis (Simmons et al., 2012; Keshari et al., 2016). Therefore, the physicochemical properties of the IPR water (e.g. pH 6.0–8.5 and nitrite 0–3 mg/l) do not appear to explain the teratogenic effects we saw. pH is an important determinant of both solubility and partitioning of ionizable organic chemicals in water. As pH decreased (from ~8.0 to ~6.0) along the treatment process it may have influenced the exposure dynamics of zebrafish to certain chemicals in the water (Fig. 3).

##### 4.3. Pathway level changes associated with IPR exposure

The biggest differences in global gene responses observed in any exposure group (and between exposure groups) were caused by differences in developmental stage. By adjusting gene expression for development, we found clear differences in global transcriptomic responses between embryos exposed to treatment water at either end of the IPR process. These differences were also associated with decreases in the amount and concentration of chemicals in the water. To illustrate, RO reduced the complexity and concentration of measured chemicals in the treated water the most (Fig. S4 and Table S7), and this was associated with a very pronounced change in global gene expression, and a complete absence of detected abnormalities in the fish compared to the previous treatment stage. This suggests that RO is an important and effective stage of IPR treatment in removing chemical contaminants and their associated toxicological effects. However,

advanced oxidation of MF treatment water (AOP1) and RO water (AOP2) significantly increased the incidence of teratogenic effects compared to RO and aquarium control water, suggesting that unknown disinfection by-products (DBP) may be generated during the UV/H<sub>2</sub>O<sub>2</sub> AOP process. Indeed, we can infer that if sufficient monochloramine is present in water post MF and/or RO, then DBP formation could occur in AOP1 and AOP2, respectively (Wang et al., 2020). There were no signs of altered expression of genes and pathways involved in oxidative stress in embryos exposed to AOP waters, suggesting that reactive oxygen species were unlikely to be responsible for the teratogenic effects observed.

#### 4.4. Pigmentation pathway responses

Having established that global gene expression was affected by exposure to water at different treatment stages, we set out to identify which genes and biological pathways had been altered, and to what extent these changes might account for the phenotypic abnormalities we had observed. As pigmentation is normally visible from 24 hpf (Kimmel et al., 1995), our discovery that some exposed embryos lacked pigmentation (even after 48 h) was of interest. Changes to pigmentation in the exposed embryos appeared in the pathway analysis of global gene expression as alterations to melanogenesis. Of the few genes that were significantly differentially expressed, somatolactin- $\beta$  (SL $\beta$ ; a glycoprotein hormone that is exclusive to fish (Wan and Chan, 2010)) stood out because of its purported role in pigmentation (Fukamachi et al., 2004, 2009; Zhu and Thomas, 1997; Nguyen et al., 2006). Significant increases in transcription of the SL $\beta$  gene occurred in embryos exposed to later treatment stages (i.e. RO, AOP2, FP and tap water), whereas embryos visibly lacking pigmentation were seen at the early stages of treatment (FE and after MF) and after advanced oxidation (AOP2) (see Table 1). Melanin Concentrating Hormone (MCH) is known to play an important role in pigmentation in animals, and is reported to have a direct inhibitory role on Somatolactin expression and release by fish pituitary cells in vitro via binding to the MCH receptor (Tanaka et al., 2009). Analysis of pigment mutants in medaka (with unique defects in proliferation and morphogenesis of certain skin chromatophores) revealed specific mutations in the somatolactin gene leading to abnormal proliferation and morphogenesis of leucophores and xanthophores (neural crest-derived pigment cells) (Kimura et al., 2014). Therefore, it is possible that the observed increases in SL $\beta$  gene transcription may have been a compensatory (negative feedback) response to the observed suppression of melanogenesis pathways in exposed embryos.

#### 4.5. Steroidogenic, neurotransmitter and retinoid pathway responses

In addition to changes in melanogenesis pathways, steroid hormone biosynthesis, retinol metabolism and tryptophan metabolism were also altered (Table 4). Indeed, two cytochrome P450 family 1 genes (Cyp1a and Cyp1b1) were significantly over expressed during the first three stages of the IPR treatment process (FE, MF and AOP1). Cyp1a expression is known to be induced by a wide variety of aryl hydrocarbon receptor agonists (Bräunig et al., 2015; Denison and Nagy, 2003; Hinger et al., 2011) and has attracted increasing interest as a biomarker of pollution due to its important role in the metabolism and elimination of aquatic contaminants in biota (Hook et al., 2014). Changes in the expression of Cyp1a may be a common factor in these responses, being an important contributor to the biotransformation of many endogenous substances, including melatonin, retinol, and various steroids (Lu et al., 2020). Certain heavy metals have also been reported to enhance basal levels of Cyp1a1 expression through transcriptional and post-translational modifications; an effect that is enhanced with co-exposure to the AhR agonist TCDD (Korashy and El-Kadi, 2005). Together these findings suggest that organic and inorganic chemicals (including AhR agonists and metals) may act together to affect

endocrine, neuroendocrine and paracrine responses through changes in Cyp1a expression.

Recent evidence links exposure to various EDCs (including chemicals that behave as estrogen or androgen agonists and antagonists) with neuroendocrine and reproductive deficits in wildlife and in humans. This is particularly apparent when exposures occur during foetal and early postnatal development (Dickerson and Gore, 2007; Gore et al., 2015, 2018). The phenotypic consequences of disruption to steroid estrogens and androgens may only become apparent during sexual differentiation and the development of secondary sex characteristics (Brion et al., 2004) and would not be apparent in the very early zebrafish embryos.

Alterations to tryptophan metabolism (see Table S6) in the first 24 h of development stood out because of its association with the synthesis of important brain neurotransmitters needed to control mood (Serotonin - anxiety and depression) and circadian rhythms (melatonin - wakefulness, sleep cycles and normal patterns of appetite). The fact that the circadian rhythm was altered at 48 hpf (Table S6) is likely to be a consequence of disruption to melatonin production. Disruptions in circadian rhythms have been recently identified as an important area of current and future research into metabolism disrupting chemicals (Mimoto et al., 2017), and may be associated with exposure to various xenobiotics, including estrogenic chemicals and aryl hydrocarbon receptor agonists (Rhee et al., 2014).

In addition to their protective role, upregulation of the Cyp450 detoxification pathways may alter tissue concentrations of retinoids resulting in hormone imbalances (Katalin and Dvorak, 2011). Retinoids behave as powerful morphogens during development, and both enhanced and repressed levels of retinoids in vertebrate tissues during development are associated with embryotoxicity and/or teratogenicity (Novák et al., 2008; Rolland, 2000) due to changes to patterns of cellular differentiation orchestrated during embryogenesis (Ross et al., 2000; Samarut et al., 2015; Niederreither and Dollé, 2008). We replicated these abnormalities, either wholly or partially, by exposing developing zebrafish to a retinoic acid (RA) receptor- $\alpha$  antagonist ER50891 thereby strengthening the association between cause and effect (Table 5). Inhibition of the RA receptor was chosen to emulate the consequences of downstream metabolic changes that might affect RA availability in tissues or possible modulation at the receptor itself. Indeed, RA is important for normal heart and coronary development (Keegan et al., 2005; Pan and Baker, 2007; Zile, 2010; Wang et al., 2018) and skeletal development (Green et al., 2016), making a plausible link between the observed cardiovascular and skeletal effects and the alterations to retinoid metabolism highlighted. Moreover, retinoid compounds, used widely in skin-lightening products and for treatment of psoriasis and acne (Orfanos et al., 1997), are known to affect pigmentation in zebrafish embryos and in vivo mammalian studies (Brannen et al., 2010).

#### 4.6. Data gaps and uncertainties

Although we can only speculate about the agents and mechanisms responsible, retinoid imbalances may occur following exposure to various organic pollutants (Novák et al., 2008) (including some pesticides, PCBs and TCDD) as well as metal contaminants (Defo et al., 2014). Interestingly, we found that metalloids, transition metals and alkaline earth metal concentrations measured in IPR water samples were associated with retinol metabolism gene expression pathways. This may be related to Cyp1a activation by metals and AhR agonists as described previously. However, as metals are widely used in a variety of personal care product (PCP) formulations (Borowska and Brzóska, 2015), we should be mindful that this relationship could occur by association of metals with chemicals that are not routinely measured in current monitoring approaches. Curiously, various components of the retinoid system play pivotal roles in mechanistic pathways associated with current disease trends in humans (such as falling fertility and rising obesity rates (Griswold et al., 2012; Keller et al., 1993; Chandra et al., 2008; Swan

et al., 2000)); trends that cannot be explained by changing lifestyle factors alone (Heindel et al., 2017). Despite this, our knowledge of the cause and consequences of real-world exposures to chemical assaults that may un hinge the retinoid system is still very much in its infancy (Inoue et al., 2010).

## 5. Conclusions

History cautions us about the need to acknowledge and respond quickly to ignorance, as well as uncertainty and risk, when protecting the environment and public health against potential new and emerging threats (European Environment Agency, 2001, 2013). Our study highlights that, despite our best efforts to use advanced treatment regimes, perturbagens are present in the water at sufficient quantities to cause biological effects during early embryogenesis in zebrafish. Identification of causative chemicals is challenging, as chemistry data for all components of these highly complex mixtures is not available. However, we show that with an intermediate molecular characterisation we can highlight relevant chemical groups and their associated biological response. In the context of environmental health our findings highlight the need for further research on the safety of advanced water treatment processes, with an emphasis on the impacts of organic and inorganic contaminants on the retinoid system.

Schematic of methodological approach and IPR process. Details of physicochemical properties of exposure waters. Approach to assessment of developmental stages and abnormalities. Illustration of abnormalities. Details (average expression, target ID, *p* values, log-odds value) of changes to the expression of individual genes in zebrafish exposed to IPR treatment waters. Minimum false discovery rate (FDR) for the loess normalised dataset using the SAM time-course analysis for gene and pathway levels. Up- and down-regulated pathways with the five lowest FDRs using the quantile normalised dataset signed area approach (with link to full pathway enrichment analysis). Detailed annotation of chemical analytical features and gene expression profiles. Changes to pesticides, EDC/Pharmaceuticals and Organics along the IPR treatment plant process. Chemical concentrations measured as part of the routine analyses of the IPR Plant by TW for the three experiments undertaken as part of this project. Summary data of abnormality data following independent developmental exposures to known chemicals. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2021.147981>.

## CRedit authorship contribution statement

ER conceived the idea with assistance from EGC. EGC provided vigilance and input to the research on an ongoing basis, and provided necessary access to the IPR facility and data. LL carried out the experimental work with the IPR waters, and PA and FF contributed to the experimental design using microarrays, and oversaw the transcriptomics. PA analysed the transcriptomic data and its association to the water chemistry data. SW exposed zebrafish to the RAR $\alpha$  antagonist and analysed the data with guidance from LL. ER interpreted the results and wrote the manuscript, in consultation with LL, PA and EGC.

## Declaration of competing interest

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.

## Acknowledgements

We thank Martin Tupper (Research Scientist) and Marie Raffin (Water Reuse Project Manager) of Thames Water Utilities Ltd. for their logistical support in enabling this research. We also thank Marie Raffin for reviewing earlier drafts of the manuscript for Thames water.

## Funding

This work was supported by Thames Water Utilities Ltd.

## References

- Agilent. One-color microarray-based gene expression analysis: low input quick amp labeling protocol. 2014, No. December.
- Arend, M.C., Pereira, J.O., Markoski, M.M., Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.-J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assunção, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.-H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliott, D., Threadgold, G., Harden, G., Ware, D., Mortimer, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J.J.E., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Contributions, A.K., Kari, G., Rodeck, U., Dicker, A.P., 2017. The zebrafish reference genome sequence and its relationship to the human genome the generation of maps used in the initial assemblies and the production of clone tiling paths were carried out. *Arq. Bras. Cardiol.* 82 (1), 70–80. <https://doi.org/10.1038/nature12111>.The.
- Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing author (s): Yoav Benjamini and Yoel Hochberg. Source: Journal of the Royal Statistical Society. Series B (Methodological), Vol. 57, No. 1 Published By: J. R. Stat. Soc. Ser. B 57 (1), 289–300. <https://doi.org/10.2307/2346101>.
- Borowska, S., Brzóska, M.M., 2015. Metals in cosmetics: implications for human health. *J. Appl. Toxicol.* 35 (6), 551–572. <https://doi.org/10.1002/jat.3129>.
- Brannen, K.C., Panzica-Kelly, J.M., Danberry, T.L., Augustine-Rauch, K.A., 2010. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. *Birth Defects Res. B Dev. Reprod. Toxicol.* 89 (1), 66–77. <https://doi.org/10.1002/bdrb.20223>.
- Braunbeck, T., Lammer, E., 2006. Fish embryo toxicity assays. *Contract* 20 (203), 725–731. <https://doi.org/10.1007/s10811-007-9182-7>.
- Braunbeck, T., Lammer, E., 2005. Draft Detailed Review Paper on Fish Embryo Toxicity Assays (UBA Contract Number 203 85 422).
- Bräunig, J., Schiwy, S., Broedel, O., Müller, Y., Frohme, M., Hollert, H., Keiter, S.H., 2015. Time-dependent expression and activity of cytochrome P450 1s in early life-stages of the zebrafish (*Danio rerio*). *Environ. Sci. Pollut. Res.* 22 (21), 16319–16328. <https://doi.org/10.1007/s11356-015-4673-6>.
- Brion, F., Tyler, C.R., Palazzi, X., Laillet, B., Porcher, J.M., Garric, J., Flammarion, P., 2004. Impacts of 17 $\beta$ -estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). *Aquat. Toxicol.* 68 (3), 193–217. <https://doi.org/10.1016/j.aquatox.2004.01.022>.
- Brookes, J.D., Carey, C.C., Hamilton, D.P., Ho, L., van der Linden, L., Renner, R., Rigosi, A., 2014. Emerging challenges for the drinking water industry. *Environ. Sci. Technol.* 48 (4), 2099–2101. <https://doi.org/10.1021/es405606t>.
- Building a Better Future <https://doi.org/10.7748/ns2014.05.28.36.58.s56>.
- Caballero-Gallardo, K., Olivero-Verbel, J., Freeman, J.L., 2016. Toxicogenomics to evaluate endocrine disrupting effects of environmental chemicals using the zebrafish model. *Curr. Genomics* 17 (6), 515–527. <https://doi.org/10.2174/138920291766616051310>.
- Chandra, V., Huang, P., Hamuro, Y., Raghuram, S., Wang, Y., Burris, T.P., Rastinejad, F., 2008. Structure of the intact PPAR-gamma-RXR- nuclear receptor complex on DNA. *Nature* 456 (7220), 350–356. <https://doi.org/10.1038/nature07413>.
- Chen, P.J., Padgett, W.T., Moore, T., Winnik, W., Lambert, G.R., Thai, S.F., Hester, S.D., Nesnow, S., 2009. Three conazoles increase hepatic microsomal retinoic acid metabolism and decrease mouse hepatic retinoic acid levels in vivo. *Toxicol. Appl. Pharmacol.* 234 (2), 143–155. <https://doi.org/10.1016/j.taap.2008.10.004>.
- Corcoran, J., Winter, M.J., Tyler, C.R., 2010. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. *Crit. Rev. Toxicol.* 40 (4), 287–304. <https://doi.org/10.3109/10408440903373590>.
- Defo, M.A., Spear, P.A., Couture, P., 2014. Consequences of metal exposure on retinoid metabolism in vertebrates: a review. *Toxicol. Lett.* 225 (1), 1–11. <https://doi.org/10.1016/j.toxlet.2013.11.024>.
- Denison, M.S., Nagy, S.R., 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol.* 43 (1), 309–334. <https://doi.org/10.1146/annurev.pharmtox.43.100901.135828>.
- Denslow, N.D., Chow, M.C., Kroll, K.J., Green, L., 1999. Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. *Ecotoxicology* 8 (5), 385–398. <https://doi.org/10.1023/A:1008986522208>.
- Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P., Waldock, M., 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.* 32 (11). <https://doi.org/10.1021/es9707973>.

- Desjardins, R., Fournier, M., Denizeau, F., Krzystyniak, K., 1992. Immunosuppression by chronic exposure to N-nitrosodimethylamine (NDMA) in mice. *J. Toxicol. Environ. Health* 37 (3), 351–361. <https://doi.org/10.1080/15287399209531676>.
- Dickerson, S.M., Gore, A.C., 2007. Estrogenic Environmental Endocrine-Disrupting Chemical Effects on Reproductive Neuroendocrine Function and Dysfunction Across the Life Cycle. , pp. 143–159 <https://doi.org/10.1007/s11554-007-9048-y>.
- Dominguez-Chicas, A., Scrimshaw, M.D., 2010. Hazard and risk assessment for indirect potable reuse schemes: an approach for use in developing water safety plans. *Water Res.* 44 (20), 6115–6123. <https://doi.org/10.1016/j.watres.2010.07.007>.
- Dong, S., Page, M.A., Massalha, N., Hur, A., Hur, K., Bokenkamp, K., Wagner, E.D., Plewa, M.J., 2019. Toxicological comparison of water, wastewaters, and processed wastewaters. *Environ. Sci. Technol.* 53 (15), 9139–9147. <https://doi.org/10.1021/acs.est.9b00827>.
- EA. Water stressed areas – final classification. 2013, No. July, 1–16. doi:LIT 8538.
- Edgar, R., 2002. Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 30 (1), 207–210. <https://doi.org/10.1093/nar/30.1.207>.
- Escher, B.L., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., Denslow, N.D., Dopp, E., Hilscherova, K., Humpage, A.R., Kumar, A., Grimaldi, M., Jaysinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraishi, F., Snyder, S., Su, G., Tang, J.Y.M., Burg, B. Van Der Linden, S.C.V. Der, Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B.H.Y., Zhang, X., Leusch, F.D.L., 2014. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays. *Environ. Sci. Technol.* 48 (3), 1940–1956. <https://doi.org/10.1021/es403899t>.
- European Environment Agency, 2013. Late Lessons From Early Warnings: Science, Precaution, Innovation.
- European Environment Agency. EEA, 2001. Late Lessons From Early Warnings: The Precautionary Principle 1896 – Late Lessons From Early Warnings: The Precautionary Principle 1896–2000.
- Fatta-Kassinos, D., Kalavrouziotis, I.K., Koukoulakis, P.H., Vasquez, M.I., 2011. The risks associated with wastewater reuse and xenobiotics in the agroecological environment. *Sci. Total Environ.* 409 (19), 3555–3563. <https://doi.org/10.1016/j.scitotenv.2010.03.036>.
- Fent, K., Sumpter, J.P., 2011. Progress and promises in toxicogenomics in aquatic toxicology: is technical innovation driving scientific innovation? *Aquat. Toxicol.* <https://doi.org/10.1016/j.aquatox.2011.06.008>.
- Fukamachi, S., Sugimoto, M., Mitani, H., Shima, A., 2004. Somatolactin selectively regulates proliferation and morphogenesis of neural-crest derived pigment cells in medaka. *Proc. Natl. Acad. Sci.* 101 (29), 10661–10666. <https://doi.org/10.1073/pnas.0401278101>.
- Fukamachi, S., Yada, T., Meyer, A., Kinoshita, M., 2009. Effects of constitutive expression of somatolactin alpha on skin pigmentation in medaka. *Gene* 442 (1–2), 81–87. <https://doi.org/10.1016/j.gene.2009.04.010>.
- Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. , pp. 1–150 <https://doi.org/10.1210/er.2015-1010>.
- Gore, A.C., Holley, A.M., Crews, D., 2018. Hormones and behavior mate choice, sexual selection, and endocrine-disrupting chemicals. *Horm. Behav.* 101, 3–12. <https://doi.org/10.1016/j.yhbeh.2017.09.001> September 2017.
- Grandjean, P., 2008. Late insights into early origins of disease. *Basic Clin. Pharmacol. Toxicol.* 102 (2), 94–99. <https://doi.org/10.1111/j.1742-7843.2007.00167.x>.
- Green, A.C., Martin, T.J., Purton, L.E., 2016. The role of vitamin A and retinoic acid receptor signaling in post-natal maintenance of bone. *J. Steroid Biochem. Mol. Biol.* 155, 135–146. <https://doi.org/10.1016/j.jsbmb.2015.09.036>.
- Griswold, M.D., Hogarth, C.A., Bowles, J., Koopman, P., 2012. Initiating meiosis: the case for retinoic acid. *Biol. Reprod.* 86 (2), 1–7. <https://doi.org/10.1095/biolreprod.111.096610>.
- Hammer, Ø., Harper, D.A.T., Ryan, P.D., 1999. PAST: paleontological statistics software package. *Palaeontol. Electron.* 4 (1), 1–9. <https://doi.org/10.1016/j.bcp.2008.05.025>.
- Heindel, J.J., Blumberg, B., Cave, M., Mächtinger, R., Mantovani, A., Mendez, M.A., Nadal, A., Palanza, P., Panzica, G., Sargis, R., Vandenberg, L.N., vom Saal, F., 2017. Metabolism disrupting chemicals and metabolic disorders. *Reprod. Toxicol.* 68, 3–33. <https://doi.org/10.1016/j.reprotox.2016.10.001>.
- Hinger, G., Brinkmann, M., Bluhm, K., Sagner, A., Takner, H., Eisenträger, A., Braunbeck, T., Engwall, M., Tiehm, A., Hollert, H., 2011. Some heterocyclic aromatic compounds are Ah receptor agonists in the DR-CALUX assay and the EROD assay with RTL-W1 cells. *Environ. Sci. Pollut. Res.* 18 (8), 1297–1304. <https://doi.org/10.1007/s11356-011-0483-7>.
- Hook, S.E., Gallagher, E.P., Batley, G.E., 2014. The role of biomarkers in the assessment of aquatic ecosystem health. *Integr. Environ. Assess. Manag.* 10 (3), 327–341. <https://doi.org/10.1002/ieam.1530>.
- Inoue, D., Sei, K., Ike, M., 2010. Disruption of retinoic acid receptor signaling by environmental pollutants. *J. Health Sci.* 56 (3), 221–230. <https://doi.org/10.1248/jhs.56.221>.
- Jane Mitchell, C.A., Kirkby, N.S., Mitchell, J.A., 2019. Eicosanoids, prostacyclin and cyclooxygenase in the cardiovascular system. *Br. J. Pharmacol.* 176 (8), 1038–1050. <https://doi.org/10.1111/bph.14167>.
- Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* 32 (17), 2498–2506. <https://doi.org/10.1021/es9710870>.
- Katalin, M., Dvorak, Z., 2011. Steroid regulation of drug-metabolizing cytochromes P450. *Curr. Drug Metab.* 12, 154. <https://doi.org/10.2174/138920011795016854>.
- Katsiadaki, I., Williams, T.D., Ball, J.S., Bean, T.P., Sanders, M.B., Wu, H., Santos, E.M., Brown, M.M., Baker, P., Ortega, F., Falciani, F., Craft, J.A., Tyler, C.R., Viant, M.R., Chipman, J.K., 2010. Hepatic transcriptomic and metabolomic responses in the stickleback (*Gasterosteus aculeatus*) exposed to ethinyl-estradiol. *Aquat. Toxicol.* 97 (3), 174–187. <https://doi.org/10.1016/j.aquatox.2009.07.005>.
- Keegan, B., Feldman, J., Begemann, G., Ingham, P., Yelon, D., 2005. Retinoic acid and heart development. *Sci. STKE* 2005 (267). <https://doi.org/10.1126/stke.2672005tw28> tw28 LP-tw28.
- Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., Wahli, W., 1993. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc. Natl. Acad. Sci. U. S. A.* 90 (6), 2160–2164. <https://doi.org/10.1073/pnas.90.6.2160>.
- Keshari, V., Adebbe, B., Simmons, A.E., Simmons, T.W., Diep, C.Q., 2016. Zebrafish as a model to assess the teratogenic potential of nitrite. *J. Vis. Exp.* No. 108, 1–6. <https://doi.org/10.3791/53615>.
- Kikuchi, K., Tagami, K., Hibi, S., Yoshimura, H., Tokuhara, N., Tai, K., Hida, T., Yamauchi, T., Nagai, M., 2001. Syntheses and evaluation of quinoline derivatives as novel retinoic acid receptor  $\alpha$  antagonists. *Bioorg. Med. Chem. Lett.* 11 (9), 1215–1218. [https://doi.org/10.1016/S0960-894X\(01\)00177-9](https://doi.org/10.1016/S0960-894X(01)00177-9).
- Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., Altman, D.G., 2013. Improving bioscience research reporting: the arrive guidelines for reporting animal research. *Animals* 4 (1), 35–44. <https://doi.org/10.3390/ani4010035>.
- Kimmel, C., Ballard, W., Kimmel, S., Ullmann, B., Schilling, T., 1995. Stages of embryonic development of the zebrafish. *Dev. Dyn.* 203 (3), 253–310. <https://doi.org/10.1002/aja.1002030302>.
- Kimura, T., Nagao, Y., Hashimoto, H., Yamamoto-Shiraishi, Y.I., Yamamoto, S., Yabe, T., Takada, S., Kinoshita, M., Kuroiwa, A., Naruse, K., 2014. Leucophores are similar to xanthophores in their specification and differentiation processes in medaka. *Proc. Natl. Acad. Sci. U. S. A.* 111 (20), 7343–7348. <https://doi.org/10.1073/pnas.1311254111>.
- Korashy, H.M., El-Kadi, A.O.S., 2005. Regulatory mechanisms modulating the expression of cytochrome P450 1A1 gene by heavy metals. *Toxicol. Sci.* 88 (1), 39–51. <https://doi.org/10.1093/toxsci/kfi282>.
- Kwong, R.W.M., Kumai, Y., Perry, S.F., 2014. The physiology of fish at low pH: the zebrafish as a model system. *J. Exp. Biol.* 217 (5), 651–662. <https://doi.org/10.1242/jeb.091603>.
- Li, C., Chen, Q., Zhang, X., Snyder, S.A., Gong, Z., Lam, S.H., 2018. An integrated approach with the zebrafish model for biomonitoring of municipal wastewater effluent and receiving waters. *Water Res.* 131, 33–44. <https://doi.org/10.1016/j.watres.2017.12.017>.
- Lu, J., Shang, X., Zhong, W., Xu, Y., Shi, R., Wang, X., 2020. New insights of CYP1A in endogenous metabolism: a focus on single nucleotide polymorphisms and diseases. *Acta Pharm. Sin. B* 10 (1), 91–104. <https://doi.org/10.1016/j.apsb.2019.11.016>.
- Martinović-Weigelt, D., Mehinto, A.C., Ankley, G.T., Denslow, N.D., Barber, L.B., Lee, K.E., King, R.J., Schoenfeld, H.L., Schroeder, A.L., Villeneuve, D.L., 2014. Transcriptomic effects-based monitoring for endocrine active chemicals: assessing relative contribution of treated wastewater to downstream pollution. *Environ. Sci. Technol.* 48 (4), 2385–2394. <https://doi.org/10.1021/es404027n>.
- McClure, M.M., McIntyre, P.B., McCune, A.R., 2006. Notes on the natural diet and habitat of eight danionin fishes, including the zebrafish *Danio rerio*. *J. Fish Biol.* 69 (2), 553–570. <https://doi.org/10.1111/j.1095-8649.2006.01125.x>.
- Menegola, E., Brocchia, M.L., Di Renzo, F., Giavini, E., 2006. Postulated pathogenic pathway in triazole fungicide induced dysmorphogenic effects. *Reprod. Toxicol.* 22 (2), 186–195. <https://doi.org/10.1016/j.reprotox.2006.04.008>.
- Mimoto, M.S., Nadal, A., Sargis, R.M., 2017. Polluted pathways: mechanisms of metabolic disruption by endocrine disrupting chemicals. *Curr. Environ. Heal. Rep.* 4 (2), 208–222. <https://doi.org/10.1007/s40572-017-0137-0> Polluted.
- Nagel, R., 2002. *DarT: the embryo test with the zebrafish *Danio rerio*—a general model in ecotoxicology and toxicology.* *Altex* 19 (Suppl. 1), 38–48.
- National Research Council, 1998. *Issues in Potable Reuse: The Viability of Augmenting Drinking Water Supplies With Reclaimed Water.* National Academies Press <https://doi.org/10.17226/6022>.
- Nguyen, N., Sugimoto, M., Zhu, Y., 2006. Production and purification of recombinant somatolactin  $\beta$  and its effects on melanosome aggregation in zebrafish. *Gen. Comp. Endocrinol.* 145 (2), 182–187. <https://doi.org/10.1016/j.ygcen.2005.09.015>.
- Niederreither, K., Dollé, P., 2008. Retinoic acid in development: towards an integrated view. *Nat. Rev. Genet.* 9 (7), 541–553. <https://doi.org/10.1038/nrg2340>.
- Novák, J., Beníšek, M., Hilscherová, K., 2008. Disruption of retinoic acid transport, metabolism and signaling by environmental pollutants. *Environ. Int.* 34 (6), 898–913. <https://doi.org/10.1016/j.envint.2007.12.024>.
- Orfanos, C.E., Zouboulis, C.C., Almond-Roesler, B., Geilen, C.C., 1997. Current use and future potential role of retinoids in dermatology. *Drugs* 53 (3), 358–388. <https://doi.org/10.2165/00003495-199753030-00003>.
- Pan, J., Baker, K.M., 2007. Retinoic acid and the heart. *Vitamin A.* vol. 75. Academic Press, pp. 257–283. [https://doi.org/10.1016/S0083-6729\(06\)75010-5](https://doi.org/10.1016/S0083-6729(06)75010-5).
- QIAGEN, 2012. *RNeasy Mini RNA Extraction.* <https://doi.org/10.1007/978-0-387-77674-3> No. June.
- R Core Team, R, 2016. *R: a language and environment for statistical computing.* R Found. Stat. Comput. 1 (2.11.1), 409. <https://doi.org/10.1007/978-3-540-74686-7>.
- Raffin, M., Germain, E., Judd, S., 2011. Optimising operation of an integrated membrane system (IMS) - a Box-Behnken approach. *Desalination* 273 (1), 136–141. <https://doi.org/10.1016/j.desal.2010.10.030>.
- Raffin, M., Germain, E., Judd, S., 2012. Assessment of fouling of an RO process dedicated to indirect potable reuse. *Desalin. Water Treat.* 40 (1–3), 302–308. <https://doi.org/10.1080/19443994.2012.671171>.
- Rhee, J., Kim, B., Lee, B., Hwang, U., Sung, Y., Lee, J., 2014. Cloning of circadian rhythmic pathway genes and perturbation of oscillation patterns in endocrine disrupting chemicals (EDCs)-exposed mangrove killifish *Kryptolebias marmoratus*. *Comp. Biochem. Physiol. C* 164, 11–20. <https://doi.org/10.1016/j.cbpc.2014.04.001>.
- Rolland, R.M., 2000. A review of chemically-induced alterations in thyroid and vitamin A status from field studies of wildlife and fish. *J. Wildl. Dis.* 36 (4), 615–635. <https://doi.org/10.7589/0090-3558-36.4.615>.

- Ross, S.A., Caffery, P.J.M.C., Drager, U.C., Luca, L.M.D.E., Sharon, A., Mccaffery, P.J., Drager, U.C., De Luca, L.M., 2000. *Retinoids in Embryonal Development*. vol. 80 (3) pp. 1021–1055.
- Ruzicka, L., Howe, D.G., Ramachandran, S., Toro, S., Van Slyke, C.E., Bradford, Y.M., Eagle, A., Fashena, D., Frazer, K., Kalita, P., Mani, P., Martin, R., Moxon, S.T., Paddock, H., Pich, C., Schaper, K., Shao, X., Singer, A., Westerfield, M., 2019. The zebrafish information network: new support for non-coding genes, richer gene ontology annotations and the alliance of genome resources. *Nucleic Acids Res.* 47 (D1), D867–D873. <https://doi.org/10.1093/nar/gky1090>.
- Samarut, E., Fraher, D., Laudet, V., Gibert, Y., 2015. ZebRA: an overview of retinoic acid signaling during zebrafish development. *Biochim. Biophys. Acta Gene Regul. Mech.* 1849 (2), 73–83. <https://doi.org/10.1016/j.bbagr.2014.05.030>.
- Schulte, C., Nagel, R., 1994. Testing acute toxicity in the embryo of zebrafish, *Brachydanio rerio*, as an alternative to the acute fish test: preliminary results. *Altern. Lab. Anim No.* 22, 12–19.
- Shi, P., Jia, S., Zhang, X.X., Zhao, F., Chen, Y., Zhou, Q., Cheng, S., Li, A.M., 2014. A cross-omics toxicological evaluation of drinking water treated with different processes. *J. Hazard. Mater.* 271, 1–8. <https://doi.org/10.1016/j.jhazmat.2014.02.007>.
- Simmons, A.E., Karimi, I., Talwar, M., Simmons, T.W., 2012. Effects of nitrite on development of embryos and early larval stages of the zebrafish (*Danio rerio*). *Zebrafish* 9 (4), 200–206. <https://doi.org/10.1089/zeb.2012.0746>.
- Swan, S.H., Elkin, E.P., F. L., 2000. The question of declining sperm density revisited: an analysis of 101 studies published 1934–1996. *Environ. Health Perspect.* 108 (10), 961–966. <https://doi.org/10.1289/ehp.00108961>.
- Tanaka, M., Azuma, M., Nejigaki, Y., Saito, Y., Mizusawa, K., Uchiyama, M., Takahashi, A., Shioda, S., Matsuda, K., 2009. Melanin-concentrating hormone reduces somatolactin release from cultured goldfish pituitary cells. *J. Endocrinol.* 203, 389–398. <https://doi.org/10.1677/JOE-09-0330>.
- Tusher, V.G., Tibshirani, R., Chu, G., 2001. SAM Method. *Stat. Med.* 20 (17), 3185–3198. <https://doi.org/10.1073/pnas.091062498>.
- Vidal-Dorsch, D.E., Colli-Dula, R.C., Bay, S.M., Greenstein, D.J., Wiborg, L., Petschauer, D., Denslow, N.D., 2013. Gene expression of fathead minnows (*Pimephales promelas*) exposed to two types of treated municipal wastewater effluents. *Environ. Sci. Technol.* 47 (19), 11268–11277. <https://doi.org/10.1021/es401942n>.
- Wan, G., Chan, K., 2010. A study of somatolactin actions by ectopic expression in transgenic zebrafish larvae. *J. Morphol.* 263 (1), 1–10. <https://doi.org/10.1002/jmor.10173>.
- Wang, S., Huang, W., Castillo, H.A., Kane, M.A., Xavier-Neto, J., Trainor, P.A., Moise, A.R., 2018. Alterations in retinoic acid signaling affect the development of the mouse coronary vasculature. *Dev. Dyn.* 247 (8), 976–991. <https://doi.org/10.1002/dvdy.24639>.
- Wang, Y., Dong, H., Wu, Z., Li, M., Li, J., Qiang, Z., 2020. UV activated monochloramine promotes metribuzin degradation and disinfection by-products formation. *Chem. Eng. J.* 385, 123846. <https://doi.org/10.1016/j.cej.2019.123846> September 2019.
- Williams, T.D., Mirbahai, L., Chipman, J.K., 2014. The toxicological application of transcriptomics and epigenomics in zebrafish and other teleosts. *Brief. Funct. Genomics* 13 (2), 157–171. <https://doi.org/10.1093/bfgp/elt053>.
- Wood, R., Long, C., Quinn, M., 2006. *Planning for Major Water and Wastewater Incidents in England and Wales No. October*.
- Zhang, K., Yuan, G., Werdich, A.A., Zhao, Y., 2020. Ibuprofen and diclofenac impair the cardiovascular development of zebrafish (*Danio rerio*) at low concentrations. *Environ. Pollut.* 258, 113613. <https://doi.org/10.1016/j.envpol.2019.113613>.
- Zhu, Y., Thomas, P., 1997. Effects of somatolactin on melanosome aggregation in the melanophores of red drum (*Sciaenops ocellatus*) scales. *Gen. Comp. Endocrinol.* 105 (1), 127–133. <https://doi.org/10.1006/gcen.1996.6810>.
- Zile, M.H., 2010. Vitamin A-not for your eyes only: requirement for heart formation begins early in embryogenesis. *Nutrients* 2 (5), 532–550. <https://doi.org/10.3390/nu2050532>.