Investigating the health effects of estrogenic chemicals individually and within wastewater treatment works effluents using an ERE-GFP transgenic zebrafish (*Danio rerio*) model.

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

.....(Ruth Ann Cooper)

Abstract

It is now well established globally that oestrogens are a major environmental contaminant in WwTW effluent discharging into receiving waterways. Even in developed countries despite advances in wastewater treatment and a general reduction in the incidence of gross point source pollution, effluent from most WwTWs contain detectable estrogenic activity; around a third of English rivers currently are thought to contain levels of oestrogenic chemicals sufficient to cause some endocrine disruption in fish. These disruptions in fish present themselves in a number of different ways including a sex ratio biased in favour of females, the presence intersex individuals; delayed sexual maturation; and alterations in reproductive behaviour. This illustrate the need for a deeper understanding on how oestrogenic chemicals act within the body to affect wildlife health.

Using a transgenic oestrogen response element (ERE), green fluorescent protein (GFP) zebrafish model, this thesis work set out to determine tissue targets and the potential for health impacts of environmental oestrogens and their mixtures within effluents from WwTWs and how these responses may vary both seasonally and for exposures for different developmental life stages. This was measured through a combination of biological endpoints including mortality/hatching rates, length, weight and condition factor (in adults), vitellogenin (*vtg*) induction (mRNA) in whole body embryos and in the livers of adults, and also through GFP quantification (measured by fluorescence microscopy on target tissues, Western blotting for whole body GFP induction and GFP mRNA induction).

The body of thesis work also sought to further establish how fluorescence responses in the transgenic zebrafish model compared in terms of sensitivity for the detection of oestrogens with vitellogenin (VTG) induction, a well-established biomarker for oestrogen exposure in fish. The ontogeny of endogenous GFP expression in individual fish was also followed from early life stages through the period of sexual differentiation using fluorescent microscopy, to determine if endogenous GFP expression could be used as a sex marker in our zebrafish model.

In the WwTW effluent studies, differences in GFP tissue patterning (and of other biological endpoints such as *vtg* induction) were found to be reflective of the varying concentrations of steroidal oestrogens found in the different wastewaters, (both temporally and between treatment works), and they also illustrated the considerable dynamic nature of the oestrogenic potencies of the WwTW effluents. For chronic effluent exposures, biological responses to both the oestrogenic effluent and the synthetic oestrogen EE2 were differed between the sexes particularly in terms of overall growth and sex differentiation parameters.

For my investigation into endogenous GFP expression, I found varying levels of fluorescence, throughout the various life stages of our zebrafish to 30dpf. There were four different patterns of GFP expression across the different body tissues recorded, based on the tissues expressing GFP and the timing and duration of that expression. Gonadal sex (measured via histology at 60dph) however was associated only with sex for one of the recorded GFP expression patterns (GFP expression in the liver at 30dph – corresponding to the female phenotype).

Overall, the results from the studies presented in this thesis illustrate the potential for differing health effects of both individual exogenous oestrogens and for a single WwTW effluent over time/season and for effluents from different WwTW. The work also highlights the importance in the timing (developmental stage) of exposure on the biological effects seen. The ability of ERE-GFP transgenic zebrafish model to allow for the analysis of individual tissues, such as the heart or brain, through non-destructive fluorescence microscopy, further demonstrates its significant advantages for screening and testing for environmental oestrogens.

Whilst these results suggest that GFP induction is less sensitive than VTG induction per se, related (in part) to the greater magnitude of response for VTG induction, there was a good positive correlation between the two variables illustrating further the utility of the GFP induction as a bio-monitor to detect for the presence of exogenous oestrogens contained in real world effluents. These findings further highlight the importance of integrating biomonitoring approaches to complement analytical chemistry techniques in the monitoring of oestrogenic chemicals in wastewater effluents.

Differences, in the levels of endogenous GFP expression across the different body tissues during early life in our zebrafish model, and prior to gonadal differentiation, are likely a reflection of the varying functional roles oestrogen receptors perform during development (i.e. they are under spatial-temporal control) and are not thought to provide an accurate marker for the determination of sex in the zebrafish model but could be utilised as a phenotypic marker for identifying females at 30dpf.

With a view towards the future, additional steps now need to be taken in order to further corroborate these findings and to maximise the potential of the transgenic ERE-GFP zebrafish model system. These include the need for identifying the molecular mechanisms of oestrogenic chemicals and of oestrogen responsive genes and pathways, particularly in the responsive target tissues identified in this thesis, to better inform on adverse health outcomes.

Ultimately, the results of this thesis further highlight how the development and application of transgenic fish models can offer huge potential for more integrative health effects assessments, allowing for an improved understanding of the risks posed by oestrogenic chemicals, and for tailoring future approaches to ERA strategies, specifically in respect of AOP frameworks.

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CONTENTS

LIST OF GENERAL ABBREVIATIONS

AR	Androgen receptor
BOD	Biological oxygen demand
BPA	Bisphenol A
COD	Chemical oxygen demand
dpf	Days post fertilisation
E1	Estrone
E2	Estradiol-17β
EE2	Ethinylestradiol-17α
EDC	Endocrine disrupting chemicals
EPA	Environmental Protection Agency
ER α	Estrogen receptor α
ER β	Estrogen receptor β
ERs	Estrogen receptors
EREs	Estrogen response elements
GFP	Green fluorescent protein
hpf	Hours post fertilisation
mRNA	Messenger RNA
NP	Nonylphenol
PCBs	Polychlorinated biphenyls
PE	Population equivalent
RNA	Ribose nucleic acid
VTG	Vitellogenin
WwTW	Wastewater Treatment works
YES	Yeast estrogen screen

LATIN NAMES OF SPECIES

Atlantic croaker	*Micropogonias undulates*
Atlantic salmon	*Salmo salar*
Channel catfish	*Ictalurus punctatus*
Chinook salmon	*Oncorhynchus tshawytscha*
Coho salmon	*Oncorhynchus kisutch*
Common bream	*Abramis brama*
Common carp	*Cyprinus carpio*
European flounder	*Platichthys flesus*
European perch	*Perca fluviatilis*
European pike	*Esox lucius*
European sea bass	*Dicentrarchus labrax*
Fathead minnow	*Pimephales promelas*
Firefly	*Photinus pyralis*
Goldfish	*Carassius auratus*
Gudgeon	*Gobio gobio*
Herring gulls	*Larus argentatus*
Japanese flounder	*Paralichthys olivaceus*
Jellyfish	*Aequorea Victoria*
Medaka	*Oryzias latipes*
Mud loach	*Misgurnus anguillicaudtis*
Northern pike	*Esox lucius*
Rainbow trout	*Oncorhynchus mykiss*
Rare minnow	*Gobiocypris rarus*
Roach	*Rutilus rutilus*
Seabream	*Sparus aurata*
Shovelnose sturgeon	*Scarphirhynchus platyorhynchus*
Threespine stickleback	*Gasterosteus aculeatus*
Zebrafish	*Danio Rerio*

CHAPTER ONE: General Introduction

1.1 A Thirsty World

The world's freshwater ecosystems are under considerable pressure as a consequence of a demand for clean water from a rapidly growing human population[1], rising economic and agricultural development, increasing waste production and pollution and the escalating threat of global climate change. Although water covers more than two thirds of the surface of our planet, only 2.5% (35 million km^3) of the total volume present is comprised of freshwater of which less than 1% (200 000 km^3) is readily available for ecosystems and direct human use, equating to only 0.01% of all water on Earth (Shiklomanov, 1993). Globally, the majority of freshwater reserves (nearly 70% or 24 million km^3) are locked away within glaciers and permanent ice sheets situated largely within the polar regions. A further 30% (8 million km^3) exists as groundwater, often located deep beneath the surface in underground basins and aquifers. In contrast, surface waters such as rivers, freshwater lakes, reservoirs and wetlands constitute only 0.3% (105,000 km^3) of the total freshwater reserves on Earth, yet are representative of around 80% of annually renewable surface and groundwater resources[2] (Connor, 2015). This is particularly pertinent in view of the rapid growth of water abstractions from rivers and lakes worldwide, which have seen a six-fold increase over the past century (Mace, 2008).

Due to the very nature of the climate of our planet, which can range from arid/semi-arid conditions to wet, tropical environments, freshwater resources are extremely variable and unevenly distributed both spatially and temporally, with less than 10 countries[3] containing 60% of the world's available freshwater supply (Aquastat, 2016). At a regional level too, there are large disparities in freshwater distribution with nearly half (44.7%) of available global supplies concentrated in the Americas, 28.9% in Asia, 15.2% in Europe, with Africa and Oceania containing a mere 9.1% and 2.1% respectively (Aquastat, 2016).

[1] Currently 7 billion people and predicted to reach 9 billion by 2050 (UNDESA, 2009*a*).

[2] Estimated at 42 750km^3 per year with considerable variation in time and space (Shiklomanov, 2000).

[3] Brazil, Russia, China, Canada, Indonesia, U.S.A., India, Columbia and the Democratic Republic of Congo.

In many countries, surface waters are the main source of freshwater abstractions - as an example, in the United States less than 20% of freshwater use is sourced from groundwater (USGS, 2016) whilst in Europe, surface waters account for around 81 % of withdrawals annually (European Environment Agency, 2018). This is comparable with countries such as Saudi Arabia, Malta and Denmark, who rely solely on groundwater as their only source of freshwater supply (Vrba and van der Gun, 2004). In addition, local variations within countries can be highly significant. Currently, in the UK, the predominant source of freshwater in central and southern areas of England is groundwater, whilst in Wales supplies are almost entirely made up of surface waters (Vrba and van der Gun, 2004). Patterns of freshwater abundance and scarcity can also potentially be further amplified by other factors such as the degree of economic development and urbanisation within a given area.

The total amount of freshwater we extract and how it is used, vary significantly from country to country and between regions. The largest amount of freshwater abstractions currently occurs in Asia, with estimated annual withdrawals of around 2451 km^3, over three times the amount of that extracted in the Americas (790 km^3) and significantly more than Europe (374 km^3) Africa (215 km^3) and Oceania (26 km^3) (Aquastat, 2016).

Globally, nearly 75% of our total annual freshwater consumption is used for agriculture, mainly through crop irrigation, with current trends indicating a 20% increase by 2050. In comparison, industry uses relatively little water (20%) with the remaining 5% used for domestic purposes (UNEP, 2016). However, due to large disparities in economic development, these amounts can vary between regions. For example, in Africa an estimated 88% of all freshwater use is for agriculture, 7% for domestic purposes and 5% for industry, with similar amounts in Asia (86%, 8% and 6% respectively). In contrast, most water use in Europe is for industry (54%) while agriculture accounts for 33% and domestic use 13% (Connor, 2015).

All in all, the balance between the amount of water we use and the amount available is growing ever more precarious and with a quarter of freshwater use today exceeding accessible supplies, its long-term availability becomes increasingly uncertain (Mace, 2008, Connor, 2015). Moreover, at the current rate of increased

usage it is estimated that by 2050 our demand for freshwater will be 70% higher than today, emphasizing the pressure (and future pressure) placed on our finite freshwater resources (MEA, 2005; World Water Assessment Programme, 2009).

1.2 Chemicals

One of the most pervasive threats occurring in freshwater ecosystems is the increasing ubiquitous contamination of chemicals. In today's progressive world there is almost no industry where chemicals are not used - they are an integral part of daily life and are commonly employed in a wide variety of products and processes. Nevertheless, the benefits which chemicals have brought to modern day society and their importance to the world economy has come at a price, as a result of the subsequent contamination of natural waterways with the estimated 300 million tons of chemical products released into aquatic environments each year (Schwarzenbach *et al.*, 2006). This is important, as reports such as the Millennium Ecosystem Assessment (MEA, 2005) recognise chemical pollution as one of the five main drivers of change (the other four being habitat loss, invasive alien species, overexploitation of resources and climate change) which negatively affect global biodiversity, one of the key factors for facilitating ecosystem stability and ensuring a continuous supply of ecosystem services such as clean water.

Currently, there are more than 121 million known organic and inorganic substances in existence globally and around 4500 newly manufactured or isolated chemicals are added to that list on an annual basis (CAS, Feb 2018). In the European Union alone, the total number of registered chemicals currently in routine daily use is estimated at around 140,000 a fraction of which have been fully evaluated to establish their effects on the aquatic environment and on human health. Of those chemicals evaluated under the EU for aquatic toxicity, 644 chemicals are classified as very toxic to aquatic life, 560 are classified as very toxic to aquatic life with long lasting effects, 315 are classified as toxic to aquatic life with long lasting effects and 204 are classified as harmful to aquatic life with long lasting effects (European Environment Agency, 2016).

In general, the types of chemicals we produce can be placed into one of the following categories:

1. Bulk or ‘base’ chemicals (including both organic or ‘petro’ chemicals and basic inorganics). These include chemicals such as methanol and ethylene (organics) and sulfuric acid and ammonia (inorganics) and serve as a starting point in the production of a number of key feedstock chemicals, which in turn are used to make tens of thousands of other chemical products.
2. Speciality chemicals (including adhesives and sealants, catalysts)
3. Agricultural chemicals (e.g. pesticides and fertilisers)
4. Human and veterinary pharmaceuticals (including antibiotics, non-steroidal anti-inflammatory drugs and hormones)
5. Metals (including lead, mercury and cadmium)
6. Biocides (including both pesticides and antimicrobials)
7. Consumer products (e.g. household cleaning products and personal care products).

In terms of production and consumption, both bulk organic and inorganic chemicals account for some of the largest amounts of chemical usage worldwide, with total annual global consumption in 2010/11 estimated at around 360 million and 740 million tonnes, respectively (Connor, 2015). Another major area of demand are the agricultural chemicals such as pesticides and fertilisers, with projected total global consumption during the same period at 4.6 million and 172 million tonnes (Zhang *et al.,* 2011; IFA, 2012). In comparison, the use of metals such as lead, mercury and cadmium (the three most commonly used metals worldwide) is significantly lower with total global output in 2010/11 estimated at 9.6 million tonnes for lead, 1,920 tonnes for mercury and 20,000 metric tonnes for cadmium (Connor, 2015). Future predictions suggest that our demand for chemicals will continue to grow, with fertiliser consumption alone forecasted to reach 193 million tonnes by the end of 2018-19 a growth rate of 2.1% per year (IFA, 2012).

Internationally, our production, use and disposal of all chemicals continue to expand, with rapid growth in developing countries in particular. As an example, over the last decade chemical production in China and India has grown at an average annual rate

of 24% and 14%, respectively, with recent predictions forecasting further corresponding rises of 66% and 59% by 2020 (UNEP, 2015). Moreover, it is projected that by the end of the decade around a third of chemical production and consumption will take place in developing countries (UNEP, 2015). In contrast, more developed countries such as the United States, Japan and Germany, have seen more modest growth rates of chemical production of between 5 and 8%, with future expansion of 22 to 25% forecasted by 2020 (UNEP, 2015).

1.2.1. Transformation Products

Once released into the aquatic environment, chemicals are subject to biotic processes such as biodegradation, or non-biotic processes, e.g. photochemical degradation or hydrolysis, which can alter their structure, resulting in changes in their physio-chemical properties (Escher and Fenner, 2011; Lambropoulou and Nollet, 2014). This can subsequently produce transformation products (TPs) or metabolites that are often more persistent and exhibit greater toxicity than their corresponding parent compounds. For example, 4-isobutylacetophenone (4-IBAP) has been found to be toxic to cultured erythrocytes and fibroblasts *in vitro* at 1 mM when compared to its parent compound ibuprofen (Cory *et al.,* 2010; Ruggeri *et al.,* 2013).

Several studies have documented the contribution of Wastewater Treatment Works (WwTW) to the widespread occurrence of TPs in the aquatic environment, whereby chemicals are often transformed from a non-biologically active form into an active form during conventional wastewater treatment processes. Examples of this include the synthetic compounds, 17α-ethinylestradiol (EE_2) and 17β-estradiol (E_2) which can enter sewage treatment works in a conjugated, inactive form but are subsequently bio-transformed into an active form via bacterial breakdown (Ternes *et al.*, 1999; Larsen *et al.*, 2008) and the surfactant nonyl-phenol (NP), a major biodegradation product of 4 nonyl -phenol ethoxylates (4-NPEOs) (Soares *et al.*, 2008). In addition, advanced oxidation processes in WwTW, such as ozonation, have also been found to transform parent compounds into by-products with toxic properties. These include many pharmaceutical products such as diclofenac and ibubrofen, industrial chemicals and fertlisers, e.g. benzotriazole and mecoprop, along with disinfection by-products such as bromate, a suspected carcinogen (Von Guten,

2003b; Fatta-Kassinos *et al.*, 2011; Knopp *et al.*, 2016). This adds further complications to chemical risk assessment.

1.2.2. Sources of chemical contaminants in the aquatic environment

The emission of chemical contaminants into our waterways can occur both directly and indirectly through a range of point source or non-point source pathways (**Fig. 1**). In general, point source contaminants can be more toxic than non-point and are related to a fixed location such as a WwTW, mining site or hazardous spill. In comparison, non-point source contaminants do not have any obvious point of entry, making them difficult to identify and therefore control, and can arise from a wide range of sources including agricultural and industrial runoff, mining, irrigation and livestock waste.

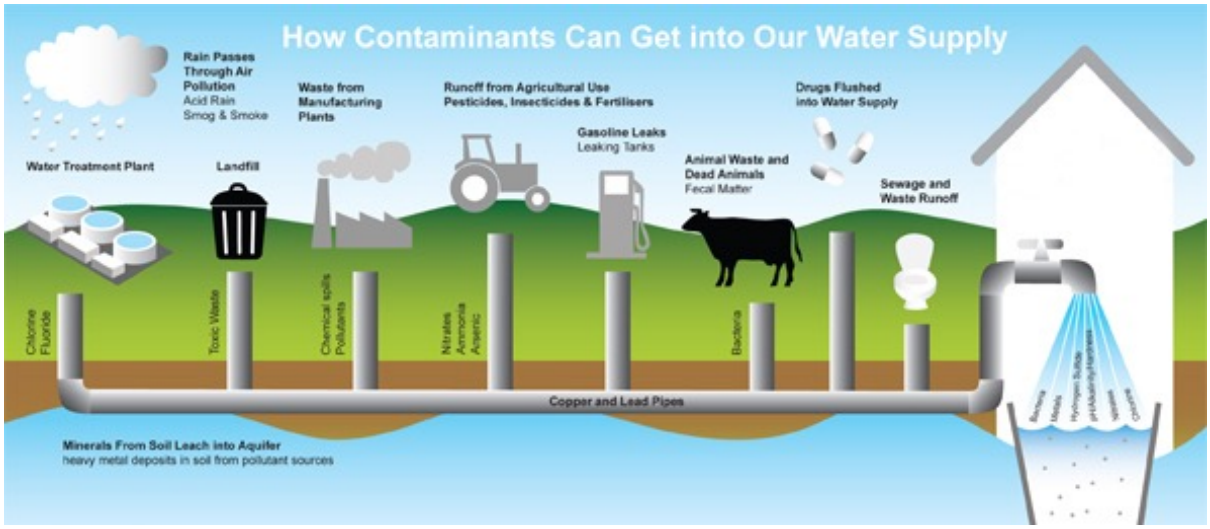

Fig. 1. Schematic diagram of the range of sources of chemical contaminants into the aquatic environment (Source: United Nations World Water Development Report 3: Water in a Changing World).

One of the major routes of chemical contaminants into the aquatic environment is via wastewater effluent discharges. Globally, it is estimated that over 450 km^3 of domestic, industrial and agricultural wastewater is released into the aquatic environment each year, which in turn requires 6000 km^3 of freshwater to treat before it can be re-used – an amount equivalent to two thirds of the worlds usable surface freshwater (Shiklominov, 1999). In most cases there is considerable dilution of these effluents in receiving waters, but in some exceptional circumstances the full flow of rivers, particularly during periods of low rainfall can be comprised entirely of treated

wastewater effluent, a phenomenon which has been observed in many countries around the world (Kumar *et al*., 2012).

The amount of wastewater produced and discharged into the planets waterways varies hugely across the world, depending on the geographical area location and/or population, with major differences between developed and developing countries. In addition, treatment processes and discharge systems can differ sharply (see section 1.4).

The potential deleterious impacts of wastewater effluents on waterways and the aquatic wildlife they contain have been reported around the world and range from eutrophication and thermal enhancement through to biological effects such as acute and chronic toxicity, reduced survivorship, growth and reproductive effects (see section 1.5). In the UK for example, levels of phosphorus (the main cause of eutrophication in England's rivers and lakes) largely originating from WwTWs (and also from agricultural run-off) was the most common reason for declines in water quality in rivers in 2016, with 59% and 26% at less than good status for fish and invertebrates respectively (EA, 2018). Furthermore, in the recent WWF Living Planet Report (2018), freshwater ecosystems were identified as the most threatened globally, with a freshwater living planet index (an indicator of the state of global biodiversity) showing an 83% decline in species populations since 1970 (WWF, 2018).

The discharge of WwTW effluents and the chemicals they contain therefore has serious implications for the future sustainable management of freshwater ecosystems. This has become of greater global concern because of the uncertainty of the long-term stability of these vital resources due to multiple stressors such as climate change and associated changes in temperature, sea levels and precipitation variability.

1.3 Wastewater Characteristics

The composition of wastewater influents received by WwTW is highly variable and consists of complex mixtures of natural and synthetic organic substances, metals and trace elements, which can originate from a variety of different sources including domestic wastewater that contains both black water (faecal matter, excreta and urine) and grey water (kitchen and bathing wastewater), storm water and other urban run-off, and wastewater generated from agricultural, industrial, horticultural and commercial businesses and institutions including hospitals and veterinary practices. As such, aquatic organisms living in waters receiving WwTW effluents are exposed to complex mixtures of chemicals with the potential for interactive (i.e. additive, synergistic, antagonistic) effects (Walker and Johnson, 1989; Silva *et al.,* 2002).

Generally, wastewater can be divided into three types: domestic, agricultural and industrial wastewater. Domestic wastewater is made up of approximately 99.9% water, with the remaining 0.1% comprising organic and inorganic, suspended and dissolved solids, together with some bacteria and microorganisms. In comparison, agricultural wastewater can contain a wide variety of pollutants, many of which are organic in nature, including fertilisers, pesticides, animal feeds and waste and veterinary medicines such as antibiotics and hormones. Some fertilisers also contain inorganic substances such as fluoride and toxic metals such as lead, arsenic or cadmium (Mullenix, 2014). Some of the largest generators of agricultural wastewaters include large livestock and poultry operations, such as factory farms and milking parlors. Similarly, industrial wastewaters are also highly variable in their chemical makeup, and often contain particular combinations of pollutants, based on the type of industry and resources processed. For example, wastewater discharges originating from pulp and paper mills can contain chloride organics and dioxins as well as suspended solids, whilst discharges from metal working industries can include oil, acids and phenols, in addition to metals such as cyanide, cadmium, lead, iron, silver and titanium (Cabrera, 2017). Some of the largest generators of industrial wastewaters include chemical plants, mining operations, pulp and paper mills, energy plants, petrochemicals and refineries and iron and steel plants. In many developing countries more than 70% of industrial wastes are dumped untreated into waters where they pollute the usable water supply (Corcoran, 2010).

1.3.1 Chemical makeup of wastewater effluents

Emissions from WwTWs contain a diverse range of different chemicals in varying concentrations, dependent on the influents received at the treatment works. In comparison, industrial and agricultural waste point source discharges tend to be more limited in their chemical makeup. Organic substances contained in WwTw effluent discharges typically include those of domestic origin, spanning pharmaceuticals e.g. EE_2, ibuprofen, ampicillin and active ingredients in personal care products (PCPs) e.g. polycyclic musks such as tonalide and antibacterial and antifungal agents such as triclosan, to chemicals derived from industrial processes, such as PCBs, phenols and solvents. Whilst many of these are commonly present at low (ng/litre to μg/litre) concentrations (Nikolaou *et al.,* 2007), nevertheless, some are of toxicological concern due to their persistent nature and/or ability to bio-accumulate in biota. These include steroidal oestrogens and alkylphenolic chemicals which have been shown to bio-concentrate up to 40,000-fold (Fenlon *et al.*, 2010; Yang *et al.*, 2014). In addition, some chemicals are biologically active even at these low exposure concentrations. Pharmaceutical products for instance, that are specifically designed to alter biological systems, can enter WwTWs as either the original compound or as metabolites, e.g. the metabolites of ibuprofen include hydroxy and carboxyibuprofen (Mazaleuskaya *et al.,* 2015). These can be further transformed during wastewater treatment (Kosma *et al.,* 2014).

There is probably not a typical WwTW effluent but in general the vast majority are composed of elaborate mixtures of natural and synthetic organic substances, metals, and trace elements. This chemical complexity makes the establishment of cause - effect relationships in aquatic wildlife inherently challenging and little is known in this regards, as traditional risk assessment is focused on single chemical effects rather than complex environmental mixtures. Moreover, comprehensive information on the toxicological properties and environmental fate and effects of most of the individual contaminants present in wastewater effluents is sorely lacking.

As many organic chemicals exist in a variety of different forms, e.g. the surfactant 4 nonyl -phenol ethoxylates (4-NPEOs) may comprise as many as 550 isomers (Guenther *et al.*, 2006) and there are numerous by-products derived from manufacturing and biotransformation, accurate quantification of the number of

chemicals found in WwTW effluents is extremely difficult to ascertain, especially given the dynamic nature of many of these chemicals as they undergo biodegradation and absorption to organic matter.

1.4 Wastewater Treatment

Wastewater treatment processes and discharge systems vary greatly worldwide, depending on the geographical area location and/or population, with major differences between countries in regulations, infrastructure and capital capacity for wastewater management, all of which will potentially have a clear and often dramatic impact on the effects of effluent discharges in receiving waters. For example, an estimated 90% of all wastewater in developing countries is discharged directly into rivers, lakes or oceans without any form of treatment (Corcoran, 2010). In particular, there are major disparities in wastewater treatment processes between developed and developing countries (**Table 1**) and among coastal and inland communities. In developing countries, less than 35% of all cities are connected to any kind of wastewater treatment system (Corcoran, 2010) - an example of this is Luanda, a city of over 4 million people in Angola, West Africa, where all collected wastewater is discharged directly untreated into the sea (Jacobsen, 2013). This is comparable to Sydney, in Australia, with a similar population, where 100% of urban wastewater is treated to some degree (Sydney Water, 2018). In many coastal regions, wastewater effluent is frequently discharged untreated directly into receiving water bodies (Corcoran, 2010).

Percentage of Sewage Treated by Region

Region	% of sewage treated
North America	90
Europe	66
Asia	35
Latin America & Caribbean	14
Africa	<1

Table 1. Proportion of sewage effluent discharge treated by global region (Source: Selman *et al.*, 2009).

Even within well developed areas such as the European Union, there are significant regional differences in the range of wastewater treatment processes with over 80% of the population from northern, central and southern countries connected to some kind of wastewater collection and treatment system in comparison to only 40% in south eastern countries (European Environment Agency, 2012). Differences in wastewater treatment practices can also be found between rural and urban areas and even within urban areas themselves. For example, many low-income households located in urban slums in developing regions such as Africa and Latin America, often experience disproportionate exclusion from basic services such as sanitation (Mara, 2003).

In the UK, over 95% of the population is connected to a WwTW, of which there are currently around 9,000 in operation nationwide. On average these works receive and process over 11 billion litres of wastewater from around 347,000 km of sewers (Environment Agency, 2012). Whilst the relatively recent advent of conventional wastewater treatment practices, which began in earnest during the middle half of the 19th century, has seen vast improvements in the control of water pollution and quality[4], problems associated with ailing, antiquated combined sewerage systems designed to collect rainwater runoff, domestic sewage and industrial wastewater in the same pipe, still in use in the majority of the UK's towns and cities, often lead to overflows of raw sewage into nearby receiving watercourses, primarily during intense storms or prolonged wet weather. In England and Wales these events occur at a rate

[4] Currently 76% of English rivers are now deemed of 'very good' or 'good' quality (Environment Agency, 2018)

of approximately 26,000 per year (Arthur *et al*., 2008). In the UK a considerable proportion of the flow of the river is often made up of treated WwTW effluent; in many English rivers this commonly equates to a 10% level of contamination and in some cases 50% of the flow of the river (Di Giulio and Hinton, 2008). In certain, exceptional cases the full flow of the river can be comprised of treated wastewater effluent during periods of low water flow or precipitation (Van Aerle *et al.*, 2002). This phenomenon is far from unusual across the rest of Europe, or indeed worldwide, with many countries experiencing similar conditions in their rivers and streams (Kumar *et al.*, 2012).

In the United States, 75% of Americans are connected to a WwTW, (approximately 16,255) with the remainder of the population served by on site decentralized or private septic systems. Typically, around 121 billion litres of wastewater are transported every day for treatment by approximately 2.3 million km of distribution system pipes (EPA, 2012). As with the UK, overflows of raw sewage from combined sewage systems regularly occur, releasing an estimated 850 billion litres of untreated wastewater into receiving waterways each year, nationally. In addition, overflows from sanitary sewage systems are estimated to occur at a rate of between 23,000 and 75,000 annually, with resultant discharges of between 11 billion and 38 billion litres of untreated sanitary wastewater (EPA, 2012).

In many developing countries, which are also some of the most heavily populated regions in the world, only a small fraction of the population is connected to a WwTW. For example, in India, home to an estimated 1.22 billion people, there are currently only 234 WwTW in operation, with a capacity to treat just 11,786 million litres (31%) of the estimated 38,254 million litres of wastewaters generated in its major cities each day (Kaur *et al.*, 2013). In the majority of African countries wastewater treatment is rare, with few cities operating any kind of WwTW at all. Even in South Africa, which has an extensive network of WwTW, of the 1,600 treatment plants operating, 60% do not meet discharge requirements (Chikanga, 2010).
In China, the most heavily populated country in the world, around 68.5 billion tons of wastewater emissions are generated each year, which is expected to rise due to rapid urbanization and industrialization, reaching 79 billion tons by 2018/19 (USDC, 2016). To deal with this high volume, the number of WwTW in operation has risen

significantly in the past decade, with around 3,340 works in operation (by 2012) with a combined treatment capacity of 142 million cubic metres per day, making China the world's second largest sewage treatment capacity after the United States. Despite this on-going construction of new facilities and the upgrading of many existing sites, many WwTWs in China fail to meet discharge standards with almost 90% experiencing problems with poor sludge treatment and disposal, and low removal efficiency rates of EDCs and nitrogen and phosphorus (Jin *et al.*, 2014).

1.4.1 Conventional Wastewater treatment

Although there are numerous types and sequences of treatment in operation in WwTW around the world, most follow a typical 3 stage process; preliminary treatment, primary treatment and secondary treatment (described below). In addition, advanced or tertiary treatments are sometimes used to remove any remaining contaminants, depending on the quality of discharge required to ensure the protection of the receiving waters (**Fig. 2).**

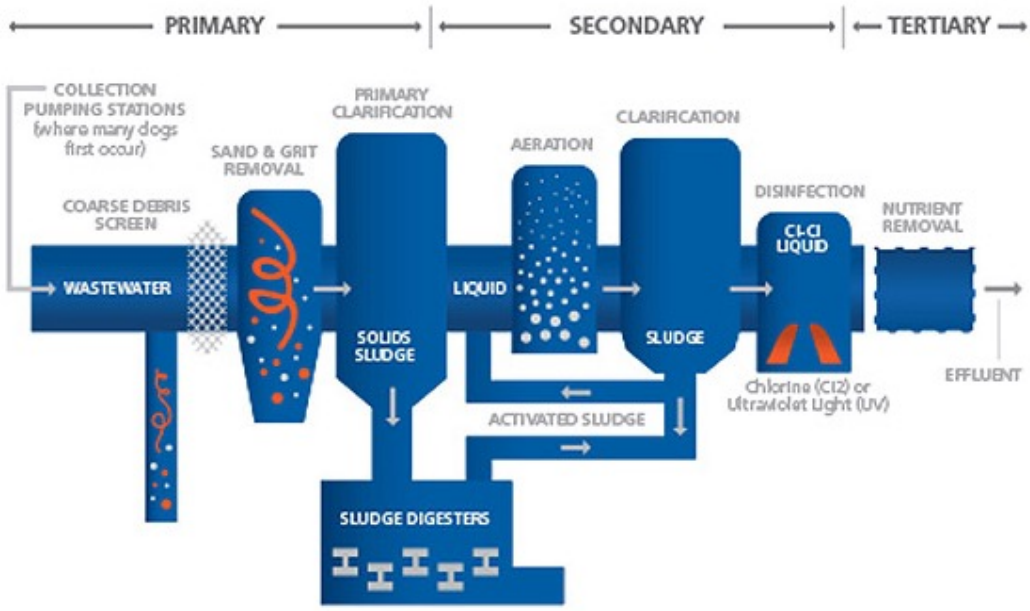

Fig. 2. Schematic diagram of a conventional wastewater treatment process (Source: Water Environment Federation (www.wef.org)).

1.4.2 Preliminary treatment

An initial step in many WwTW is the removal or screening of large debris (> 25-30mm) such as wood, plastics, cloth materials and paper. This is done through mechanical screens with the primary purpose of ensuring the removal of large items that might otherwise block pipe or mechanical systems within the treatment works. Smaller inert solids (i.e. sand and gravel) are removed through finer mesh screens. Any retained material is then normally cleaned and compressed for disposal to landfill or for incineration.

1.4.3 Primary treatment

After preliminary treatment, the next step in the treatment process is to submit the wastewater to a process of sedimentation. During this phase sewage passes into large quiescent tanks called primary clarifiers, where flow rates are lowered to allow the separation of any remaining heavy organic solids. Suspended particles settle to the bottom of the tank to form thick slurry known as primary sludge, whilst any floating material such as grease and plastics rises to the surface and is subsequently skimmed off. Retention time in these tanks varies but is usually a minimum of 2 hours to facilitate the settlement of suspended solids. Remaining residual screened wastewater, or settled sewage, is then taken forward for secondary treatment.

1.4.4 Secondary treatment

Following primary treatment, wastewater undergoes secondary (biological) treatment, a key step in the removal of organic carbon. This process involves an aerobic biological treatment stage, whereby microorganisms are used to break down and remove most of the remaining dissolved and suspended organic material and, ideally, allow the release of carbon dioxide. In general, this process consists of two different forms; biological filtration (BF; also termed tricking filters) and conventional activated sludge (CAS) tanks. Other processes commonly used include rotating biological contacters (RBC), biological aerated flooded filter tanks (BAFF) and continuously operated upward flow filters (COUFF). The WwTWs studied in this thesis employ either CAS or BF secondary treatment processes which are described in more detail below.

1.4.4.1 Biological filter treatment

During biological filtration, pre-treated wastewater is trickled vertically via rotating sprinklers into large tanks filled with a bed of solid media. The type of media used at an individual treatment works is often affected by which materials are available locally and can include rock, gravel, blast furnace slag or specially fabricated plastics. These materials are used as they are highly permeable and have considerable internal surface area in addition to a very high surface area per unit volume, which allows for effective microbial attachment, wastewater retention time, air flow and durability. As the wastewater flows over the media, microorganisms already in the water gradually attach themselves and form a biological film. Passage of the wastewater over the media also provides dissolved oxygen, which is required for the biochemical oxidation of organic compounds, and releases carbon dioxide gas, water and other oxidized end products. As the biological film thickens through microbial growth, oxygen cannot penetrate its full depth, creating anaerobic organisms near the surface, in which denitrification can occur. Eventually, the biological film sloughs off into the liquid flow and is gathered up by an underdrain system, where it is subsequently transported to a clarifier or secondary sedimentation tank. This then produces a relatively clear effluent which can either be discharged or sent on for advanced treatment.

1.4.4.2 Activated sludge treatment

Developed in England in the early 1900s, the activated sludge system utilises a microbial culture of bacteria and other micro-organisms, known as activated sludge, which feed on the organic material in the wastewater. The resultant product known as mixed liquor is agitated and aerated in large tanks and subsequently allowed to settle. Following this procedure, treated wastewater is then sent to final settlement tanks, separated from the activated sludge and subsequently passed through to an outfall pipe for final discharge into the aquatic environment or for further advanced treatment. Any remaining activated sludge (or ‘secondary sludge’) is then either returned for re-use or combined with sludge from the primary treatment process for separate treatment and disposal.

Whilst both BF and CAS processes provide the necessary bacteria and oxygen for a natural purification of wastewater effluent, in general, CAS processes have higher

removal efficiencies (95-98%) of organic matter compared with BF (80-85%) (Eckenfelder *et al.*, 2014). However, the main disadvantage of CAS treatment, namely the production of high amounts of excess sludge, mean that around 50–60% of the total costs of wastewater treatment can be spent purely on the treatment and disposal of sludge (Eckenfelder *et al.*, 2014). In addition to removing most polluting organic matter, secondary biological treatment can, where required, eliminate most of the nitrogen and phosphorus in sewage, therefore reducing the nutrient load on any receiving waterways (Metcalf and Eddy, 2004).

Treatment of sludge derived from any of the above methods can take the form of several different processes, dependent of the intended final use, and may include anaerobic and aerobic digestion, de-watering, lime stabilisation, thermal drying and incineration. Treated sludge (usually known as bio-solids) is a valuable product of wastewater treatment works as it contains organic material and nutrients that are important for agriculture or land reclamation. The organic material is also a source of carbon and can be re-used as a fuel to produce energy. However, the sludge materials generated can be contaminated by hazardous substances, such as pharmaceuticals (Giger *et al.*, 2003) and personal care products (Brausch and Rand, 2011) from conventional WwTW, chloride organics and dioxins from paper mill effluents and growth promoting anabolic hormones in cattle feedlots (Motoyama, 2011).

1.4.5 Advanced treatment

Whilst conventional treatment processes are relatively effective in removing some of the organic chemicals found in wastewater effluents, many recalcitrant organic compounds are often only partially degraded during treatment and as such are frequently discharged at appreciable levels into receiving water bodies. In order to improve the quality of an effluent before it is discharged into a receiving waterway, additional treatment steps are sometimes included for further purification, particularly if the treated wastewater is to be re-used immediately thereafter. In most cases, this advanced treatment follows secondary treatment and is commonly referred to as tertiary treatment. However, advanced treatment processes can sometimes be combined with primary or secondary treatment (e.g. addition of chemicals to primary

clarifiers or aeration basins to remove phosphorus) or used in place of secondary treatment (e.g. overland flow treatment of primary effluent).

1.4.5.1 Membrane filtration

Membrane filtration is a process whereby suspended or colloidal contaminants, typically smaller than 10^{-2} mm, are removed using a high-pressure gradient which forces wastewater through a selective barrier or semi permeable membrane. Chemicals are removed by a combination of different methods including size exclusion, adsorption and charge repulsion.

Various types of membranes, usually derived from organic materials such as cellulose, polypropylene or acetate are available, which range in pore size, thickness and filtering capacities, and this consequently affects the efficiency of contaminant separation (Metcalf and Eddy 2004). There are four basic membrane filtration processes; reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF) and microfiltration (MF). In MF and UF processes, the separation of particles occurs largely by straining (sieving) in comparison to NF and RO, where separation is achieved primarily by diffusion. Any material (or concentrate as it is commonly referred to) which does not pass through the membrane, including any removed contaminants, is subsequently treated or disposed of separately or can be re-circulated for further use in the feed water. Several studies have shown that smaller pore sized membranes (RO & NF) can reliably remove many EDCs and other trace organics from wastewater (Snyder *et al.*, 2007). In some instances, removal rates as high as 90-95% can be achieved in comparison to larger sized membranes (MF & UF) which do not perform as well (Huang and Sedlak, 2001; Nghiem *et al.*, 2004; Kim *et al.*, 2007) suggesting that size exclusion plays a significant role in overall efficiency. In view of this, membrane filtration is gaining widespread use as both a pre-treatment or post-treatment option in wastewater management. Moreover, this type of system has several other benefits including less use of chemicals, low space requirements, higher efficiency & lower energy consumption levels and the ability to filter and disinfect in a single step (without the need for subsequent de-chlorination). However, shortcomings such as the requirement for regular maintenance/cleaning and the susceptibility of membranes to fouling which makes the process less efficient

are also worth noting (Yangali-Quintanilla *et al.,* 2009) as are the high running costs of both RO and NF systems, which also generate a concentrated reject stream that requires additional treatment.

1.4.5.2 Adsorption

Adsorption works on the principal of adhesion, that is, the tendency for dissimilar particles or surfaces to adhere to one another. In the case of wastewater treatment, this is achieved through an activated carbon treatment process, whereby the molecules of a dissolved substance accumulate or adhere to the surface of an adsorbent solid, in this case carbon (Rashed, 2013). Two different sizes of activated carbon are typically used in wastewater treatment; Granular Activated Carbon (GAC) and Powered Activated Carbon (PAC). Of these, GAC is the most widely applied treatment and consists of larger particle sizes than PAC (generally in the order of 1 mm or more) packed into columns, which can be regenerated after use, through which wastewater is passed. In comparison, PAC is made up of smaller particles which are directly added to an anaerobic or aerobic treatment system, which must subsequently be removed after use and disposed of (Rashed, 2013). Both GAC and PAC have been found to be very efficient in removing trace hydrophobic organics and conventional micro-pollutants such as pesticides from wastewater (Serrano *et al.,* 2011; Margot *et al.,* 2013). Currently, research is focused on the use of activated carbon for removing EDCs, with varying results suggesting that removal efficiencies can vary dependent on the physicochemical properties of the contaminants (Rashed, 2013).

As with membrane filtration, adsorption systems require little space and can also be easily incorporated into existing WwTW facilities. The high surface area to volume ratio also allows for effective removal of a wide range of contaminants (Rashed, 2013). Disadvantages in activated carbon treatment processes include higher running costs and energy demands (= high carbon footprint) and a greater degree of mechanical failure. Variations in pH, temperature & flow rate can also affect the adsorption process (and hence efficiency). In addition, the subsequent production of carbon monoxide as a by-product of the process must be removed (through

afterburners & scrubbers) and either disposed of or regenerated, as is the case for any spent carbon (Martınez *et al.*, 2006; Rashed, 2013).

1.4.5.3 Advanced Oxidation Processes

The removal of contaminants from wastewater by advanced oxidation processes (AOPs) is based on the generation of hydroxyl radicals (OH) a group of highly reactive and subsequently short-lived chemical oxidants, with the ability to oxidize almost any organic and some inorganic compounds, producing CO_2 and inorganic ions. Hydroxyl radicals are generated with the aid of one or more primary oxidising agents, e.g. Ozone (O_3), Chlorine dioxide (ClO_2) or Hydrogen Peroxide (H_2O_2), catalysts such as iron ions or metal oxides, and irradiation (e.g. UV light, solar light, ultrasound) either separately or in combination (Stasinakis, 2008).

As with other advanced treatments, there are both advantages and disadvantages with AOPs, dependent on the type of oxidant and irradiation employed. For example, with ClO_2 oxidation systems, no halogenated disinfection by-products are formed, which have previously been associated with an increased risk of bladder cancer (Villanueva *et al.*, 2004) and adverse reproductive effects, e.g. premature births, reduced semen quality and stillbirths (Nieuwenhuijsen *et al.,* 2009), thus making it a commonly used treatment in the purification of drinking water. In addition, ClO_2 is extremely effective in inactivating a large number of harmful pathogens and can disinfect and sanitise in seconds compared to other micro-biocides which may take many hours or even days to work (Vaezi *et al.*, 2004). Similarly, with O_3 oxidation, fewer disinfection by-products are generated (in particular, chlorinated by-products) and most organic and inorganic material is eliminated, including many pathogenic microorganisms. Moreover, O_3 can be generated on site as needed, subsequently decomposing to O_2 as it reacts with other substances, making it a more environmentally friendly alternative to oxidation with chlorine and other advanced treatments such as absorption (activated carbon) or membrane filtration i.e. reverse osmosis (Gong *et al.,* 2008).

The main disadvantages of AOPs are the high operation costs and energy demands (= high carbon footprint), with both O_3 and UV oxidation requiring specialised

equipment (i.e. chemical reagents and lamps) and regular maintenance; in the case of UV systems, to produce a highly treated final effluent, any solids must be frequently removed to ensure that target microorganisms are not shielded from the UV radiation (Esplugas *et al.,* 2007). Conversely, several studies have also shown that some AOPs such as ClO_2 and O_3 can still generate toxic oxidation by-products such as N-nitrosodimethylamine (NDMA) a semi-volatile highly mutagenic compound that is suspected of carcinogenic activity in the human body and 4-ethylbenzaldehyde and 4-ethylbutyl benzene, both by-products of the pharmaceutical ibroprofen (Andrzejewski *et al.*, 2005, Haoping *et al.,* 2015).

Of the WwTW studied in this thesis, only Exeter and Plymouth use advanced treatment processes, with both works employing UV oxidation methods.

1.4.5.4 Constructed wetlands/reed-beds

Constructed wetlands/reed-beds are engineered, biological treatment systems, built to recreate the structure and function of natural wetlands, by the employment of plants, soil and associated microbial communities to remove or transform a wide range of pollutants found in wastewater effluents.

There are several types of constructed wetlands which can be categorized according to three main criteria, (1) hydrology (presence of open water-surface or subsurface flow), (2) flow path (horizontal or vertical) and (3) macrophytic growth (emergent, submerged, free-floating) (Vymazal, 2008). In addition, different types of constructed wetlands may be combined with each other (i.e., hybrid or combined systems) to utilize the specific advantages of the different systems.

Both constructed wetlands and reed-beds are relatively inexpensive to design & construct and are easy to operate & maintain (i.e. self-sustaining) and therefore do not require highly trained staff. They also provide effective, reliable and ecologically sound wastewater treatment with no sludge production and can accommodate both large and small volumes of wastewater and varying contaminant levels. In addition, any final effluent can be used for irrigation (where appropriate) due to high-nutrient and low pathogen content. Conversely, whilst constructed wetlands have been

shown to be effective in removing pathogens and most pharmaceuticals and personal care products (PPCPs) from wastewater (Conkle, White, and Metcalfe, 2008; Matamoros *et al.,* 2008; Dordio *et al.,* 2009) they are far less effectual at removing heavy metals. Effluent from some types of constructed wetlands (i.e. lagoons) can also contain algae and therefore require additional treatment to reach required discharge standards. In addition, both types of advanced treatments require more operating space and therefore take up more land than other treatment systems (e.g. membrane filtration and adsorption). They can also be less efficient in colder climates and prone to seasonal effects, i.e. nutrient removal takes place mainly during the growing season (Wang *et al.,* 2017).

1.5 Evidence for health impacts of wastewater effluents

The impact of the discharge of wastewater effluents into the world's rivers, lakes or oceans, has been widely linked to adverse effects on the environment and on aquatic wildlife health, both at an individual and population level. Worldwide, one of the most pervasive water quality problems is eutrophication, a consequence of excessive nutrient loads (largely nitrogen and phosphorus) into surface waters, which often lead to excessive plant growth and algal blooms, with subsequent effects including oxygen depletion, increased turbidity and toxicity (Yang *et al.,* 2008). In particular, eutrophication has been closely related to the development of 'dead zones' in coastal regions, with one of the largest (up to 20 700 km^2) found in the Gulf of Mexico, which receives large levels of fertilizers from the Mississippi River basin (Rabalais *et al.,* 2002). It has also been linked to population level effects, such as the collapse of the Baltic Sea cod fishery in the early 1990s, which occurred due to oxygen depletion, which subsequently interfered with the development of cod eggs (UNEP, 2015).

Another latent effect of wastewater effluent is thermal enhancement. As all aquatic wildlife have an archetypical temperature preference and tolerance limit (Burton *et al.* 2018), any changes in the thermal regime of a water body can potentially have a major impact on important biochemical and physiological activities, as well as the ecological stability of that environment. Some effluents, such as cooling water discharges from industrial manufacturing and power plants, are considerably warmer

than receiving waterways and can therefore increase ambient temperatures, reducing the availability of dissolved oxygen. This can lead to a wide range of direct and indirect effects, including reproductive disruption (Luksiene and Sandström, 1994; Luksiene *et al.,* 2000) alterations in immune function (Wiklund *et al.,* 1996) reduced species abundance and diversity and increased mortality (Wellborn and Robinson, 1996). Conversely, a rise in water temperature may actually be of benefit as demonstrated by an increase in growth rate in perch *(Perca fluviatilis)* populations exposed to cooling water releases from a nuclear power plant in Sweden, which resulted in a marked increase in the abundance and survival of juveniles (Luksiene and Sandström, 1994) although there are confines here too as this may favour particular species more than others, leading to an eventual negative impact on biodiversity.

Significant evidence from both field and laboratory studies have also shown a clear link between exposure to wastewater effluents and potentially deleterious health effects in aquatic wildlife, including genotoxic damage (Gravato and Santos 2003; Liney *et al.,* 2006), alterations in immune function (Price *et al.,* 1997; Hoeger *et al.,* 2005; Liney *et al.,* 2006), altered activity of hepatic phase I/phase II biotransformation enzymes (Gravato and Santos 2003; Hoeger *et al.,* 2005; Gagne *et al.,* 2006), nephrotoxicity (Liney *et al.,* 2006), and reduced growth/condition (Smolders *et al.,* 2002).

In particular, exposure to WwTW effluents has been associated with endocrine disrupting effects such as the induction of intersexuality (Vajda *et al.,* 2008; Williams *et al.,* 2009; Bahamonde *et al.,* 2015a/b) disruptions to hormone levels (Sepulveda *et al.,* 2002; Hoger *et al.,* 2006; Jeffries *et al.,* 2008) and reduced gamete production and fertility capability (Jobling *et al.,* 2002a, 2002b). Historically, most of the available literature on the endocrine disrupting effects of WwTW effluents has focused on two main classes of chemicals, namely, estrogenic or anti-oestrogenic (chemicals that mimic or block natural oestrogens) and androgenic or anti-androgenic (chemicals that mimic or block natural testosterone) activity and their effects. Chemicals such as these which can disrupt the endocrine system are known as endocrine disrupting chemicals (EDCs).

As the majority of the work of this thesis is focused around oestrogenic chemicals (a known class of endocrine disruptors) and their biological effects, I will next touch on endocrine disruption and examine the evidence for adverse health effects of commonly found EDCs in wastewater effluent.

1.6 The endocrine system

The endocrine system is one of the major systems in the human body responsible for the regulation, co-ordination and maintenance of a number of physiological mechanisms including growth, reproduction, development, and many of the processes of metabolism. It is composed of a series of ductless glands (e.g. pituitary, thyroid, testes, ovaries, hypothalamus, pancreas and adrenal glands) which release chemical messengers – hormones – secreted by endocrine cells, generally at very low concentrations, directly into the bloodstream which are then transported throughout the body, eliciting responses only in target cells (Marieb, 2014). Many additional organs, such as the heart, liver, intestines and kidneys all possess secondary endocrine functions and also secrete hormones.

There are two main classes of hormones: the steroid hormones (e.g. cortisol, estradiol and testosterone) and the non-steroidal hormones which include amines such as epinephrine and norepinephrine, polypeptides such as oxytocin and vasopressin, proteins such as growth hormone and insulin, and glycoproteins such as Follicle Stimulating Hormone [FSH] and Gonadotrophin Releasing Hormone [GnRHS]) (Neave, 2008).

In vertebrates, steroid hormones are relatively small, lipophilic molecules produced in the gonads and placenta (sex steroids), and in the adrenal cortex (corticosteroids). The molecular size of a chemical, which can be expressed in terms of weight (MW) and volume (V), is an important metric in terms of its bioavailability within a living system. As a rule, when molecular size increases, toxicity and bioavailability decreases (Kostal, 2016). This is because smaller molecules (termed as those with an upper MW limit of 900 kDa) are able to rapidly diffuse freely across cell membranes, without the involvement of membrane bound receptor proteins and hence reach intracellular sites of action (Macielag, 2012).

There are various guidelines for evaluating the bioavailability of a chemical, one of which is known as Lipinski's 'rule of five', which was developed to screen out pharmaceutical products with probable low absorption rates. As part of the rule of five, a MW no greater than 500 kDa is recommended for oral small molecule drug candidates, however, this is limited to those which are taken up through passive transport mechanisms (Lipinski, 1997; Macielag, 2012). As well as pharmaceutical products, many other biologically active chemicals which are commonly detected in the aquatic environment, possess a MW less than 500 kDa, such as the plastic bisphenol A (BPA) and alkylphenols such as nonylphenol, with MWs of 228 and 220 kDa respectively.

In contrast, non-steroid hormones, which are water-soluble and therefore unable to diffuse into cells, exert their effects by binding with specific receptor molecules on the target cell membrane. This then causes a second messenger (i.e. cyclic nucleotide, calcium [Ca] ion) to be released within the cell. Once in the nucleus, the hormone-receptor ligand complex then binds to specific DNA sequences and induces transcription of its genes (synthesis of mRNA) (Neave, 2008).

The gonadal hormones, or sex steroids, can be divided into three main groups; the oestrogens (Estradiol-17β [E_2], the predominant oestrogen in vertebrates, Estrone [E_1] and Estriol [E_3]) which are involved in feminine development and in male reproductive function, the progestins (e.g. progesterone) which are involved in maturation of the mammalian oocyte and the androgens (e.g. testosterone) which are involved in male development (Brevini *et al.,* 2005; Thomas *et al.,* 2006). In vertebrates, sex steroids are synthesised via a pathway involving the sequential degradation of cholesterol to progestins, then subsequently to androgens and finally to oestrogens. Oestrogens are mainly produced in the follicular cells of the ovaries and in the placenta and in small amounts by the adrenal glands and within the Leydig cells of the testes (Whitehead and Nussey, 2001).

Sex steroids function by exerting a negative feedback effect on the hypothalamus, hence regulating the release of gonadotrophins by the pituitary gland, which in turn increases or reduces the initial stimulus, thereby inhibiting its ability to continue. An example of this is the ovarian cycle in females, whereby the release of progesterone

inhibits the production of GnRH, which in turn stops the maturation and release of an ovum (Whitehead and Nussey, 2001).

1.6.1 Endocrine disrupting chemicals

Endocrine disrupting chemicals (EDCs) are defined as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process." (Environmental Protection Agency, 1996).

Due to their ubiquitous occurrence in the aquatic environment (largely through WwTW effluent discharges) and potential to adversely affect the health of aquatic life from various taxonomic groups, EDCs have emerged as a major area of scientific research worldwide. As the endocrine system is highly conserved among vertebrates, with a few known similarities in some invertebrates, it is extremely probable that most species[5] are vulnerable to the potential deleterious effects of EDCs. As such, they are frequently classed as priority substances with regards to water pollution. For example, under the European Union Strategy for Endocrine Disruptors, a total of 118 chemicals are currently considered to be priority substances, as either persistent in the environment or produced at high volumes (EU, 2012).

EDCs are extremely heterogeneous in structure (Swedenborg *et al.,* 2009) and are made up of a broad and diverse range of biologically active compounds including synthetic organic compounds such as pesticides (e.g. organophosphates, methoxychlor, dichloro-diphenyl-trichloroethane or DDT), fungicides (vinclozolin) pharmaceutical agents (diethylstilbestrol, DES), heavy metals (e.g. lead, mercury, cadmium) dioxins, plasticizers (phthalates), plastics (bisphenol A or BPA), polychlorinated biphenyls (PCBs), flame-retardant polybrominated diphenyl ether (PBDE), and antifoulant paint additive (tributyltin), alkylphenols (e.g. nonylphenol and octylphenol) as well as natural plant-derived EDCs termed phytoestrogens (e.g. β-

[5] A review by Miyamoto and Burger (2003) noted over 200 species which are either known or suspected to have been affected by EDCs, including examples from all five major vertebrate classes, and from at least two invertebrate phyla.

sitosterol, isoflavoids, lignans) and natural and synthetic hormones (e.g. 17β-estradiol, 17α-ethinylestradiol). Adverse effects of around 800 chemicals with endocrine disrupting properties, that are used on a daily basis worldwide, have been shown in both humans and wildlife (UNEP, 2015).

One of the major issues in the study of EDCs is the seeming complexity of their mechanisms of action, which may occur through multiple signaling pathways and targets. Some chemicals can mimic natural hormones and are therefore capable of binding directly with and activating hormone receptors, acting as a hormone receptor agonist, thereby stimulating specific physiological processes such as reproduction and development. Others block the receptor so it is unavailable to the natural ligand, thus acting as a hormone receptor antagonist. Moreover, some EDCs are able to interfere with more than one type of hormone receptor, though usually with varying affinities, e.g. the insecticide DDT (dichloro-diphenyl-trichloroethane) a known estrogen agonist, can also be metabolised into the androgen antagonist DDE (Dichlorodiphenyldichloroethylene) (Rasier *et al.,* 2007).

EDCs have also been found to alter hormone synthesis and or/metabolism, with some acting as aromatase inhibitors, thus effecting the conversion of androgens to estrogens by cytochrome P450 aromatase (Swedenborg *et al.,* 2009). In fish, the potential for aromatase inhibitors to disrupt sexual differentiation has been demonstrated in a number of studies, in several different species. Treatment of Japanese flounder (*Paralichthys olivaceus*) with the anti-estrogen tamoxifen (used in the treatment of breast cancer) by Kitano *et al.,* (2007) caused the sex-reversal of genetic females to phenotypic males (at concentrations of 100 ug/g diet) with similar results in the same species and in chinook salmon (*Oncorhynchus tshawytscha*) and zebrafish after exposure to the clinical aromatase inhibitor fadrozole (Piferrer *et al.,* 1994; Anderson *et al.,* 2004; Kitano *et al.,* 2007).

Other mechanisms of EDC action have also been reported, in particular with involvement of the arylhydrocarbon receptor AhR, a ligand-activated transcription factor and key regulator of cellular responses to exposure to environmental pollutants. Several studies have shown that AhR is strongly activated by many EDCs including dioxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD),

polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCB), as well as polycyclic aromatic hydrocarbons (PAHs) such as 3-methylcholanthrene (3-MC), benzo[a]pyrene (BaP) and benzoflavone (Poland and Knutson 1982; Denison and Heath-Pagliuso, 1998).

EDCs also challenge long-established concepts in toxicology, particularly the dogma of "the dose makes the poison," as they are able to elicit effects at low concentrations (i.e. doses below those traditionally used in toxicological studies or where any biological change occurs in the range of typical human exposures (Melnick *et al.,* 2002) that are not predicted by effects at higher concentrations. In addition, many EDCs can produce a nonlinear relationship between concentration and effect i.e. a non-monotonic dose-response curve (Vandenberg *et al.,* 2012).

Although EDCs have been shown to affect every possible cellular hormonal pathway, the majority of available information concerns their interference with the hormone receptors of the structurally related nuclear receptor (NR) super-family. The NRs function as ligand-inducible transcription factors that mediate the expression of target genes in response to ligands specific to each reporter (reviewed in Gronemeyer *et al.,* 2004)**.** Comparisons of the number of identified NR genes in vertebrates varies between species, with between 47-48 NRs identified in human, mouse/rat and dolphin genomes, which are involved in vital functions such as homeostasis, reproduction, development, metabolism, and responses to xenobiotic substances (Bunce and Campbell, 2010). In comparison, in some teleost fish additional NRs have been identified including 73 and 74 NRs in zebrafish and tilapia, respectively (Schaaf, 2017). These additional genes are mainly due to the existence of paralogue genes within the same genome, e.g. in zebrafish, two or more paralogues correspond with one of 20 NRs in humans (Zhao *et al.,* 2015). The hormone receptor members of the NR family include the steroid hormone receptors (ERs), androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor as well as the thyroid hormone receptors (TRs).

1.6.2 Oestrogen receptor function and regulation

The classical (direct) pathway for oestrogen signaling involves ligand activation whereby ligand-bound ERs in the cell nucleus dimerise and bind to oestrogen response elements (EREs) located within the upstream regulatory region (promoters) of target genes (Nilsson *et al.,* 2001; Bjornstrom and Sjoberg, 2005). As both ER subtypes are co-expressed in many cell types, the receptors may form ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers. In addition, gene regulation by indirect DNA binding can also occur i.e. protein-protein interaction with other transcription factors such as Fos/Jun (AP-1-responsive elements) after ligand activation (Kusher *et al.,* 2000; Saville *et al.,* 2000). Other known molecular pathways include a ligand-independent pathway, such as growth factor signaling, which leads to activation of kinases that may phosphorylate ERs and thereby activate them to dimerize, bind DNA and regulate genes (Kato *et al.,* 1995; Simoncini *et al.,* 2004). Increasing evidence also suggests that ERs can signal by regulating several different signaling cascades in a fast 'non-genomic' fashion. For example, the G-protein coupled receptor GPR30, has been shown to bind E_2 and initiate subsequent signaling cascades (Langer *et al.,* 2010). However, current data for this alternate signaling mechanism remains limited and controversial, for any model system (Razandi *et al.,* 1999; Soltysik and Czekaj, 2013).

In mammals, there are two known subtypes of oestrogen receptors involved in cell signalling, usually referred to as ERα and ERβ, each encoded by a separate gene, ESR1 and ESR2 respectively. As with most other members of the NR family, ERs contain highly conserved, distinct domains. The central and most conserved domain is the DNA-binding domain (DBD) - with approximately 97% homology - which mediates specific interaction with target DNA sequences (Heldring *et al.,* 2007). Located in the C-terminal - with approximately 56% homology - the ligand-binding domain (LBD) is responsible for ligand recognition, dimerization, interaction with coactivators and ligand-dependent transcriptional activation (Heldring *et al.,* 2007). In comparison, the NH_2-terminal domain is poorly conserved (only 24%) and represents the most variable domain both in sequence and length (Kuiper *et al.,* 1997; Paech *et al.,* 1997). A small group of orphan nuclear receptors - known as oestrogen-related receptors (ERRs) - also share some target genes with conventional nuclear ERs. ERRs are known to bind to oestrogen response elements (EREs) and modulate transcription but are not believed to be directly activated by oestrogens. They also

have their own specific response elements (Giguere *et al.,* 1988).

In humans, tissue specific expression of ERα and ERβ is well characterised, with both subtypes found in a wide variety of tissues (Ascenzi *et al.,* 2006; Koehler *et al.,* 2005). In some cases, both ERs are expressed at similar levels, while in others, one or the other subtype predominates. In addition, both ERs may be found in the same tissue but in different cell types. ERα is mainly expressed in the uterus, mammary gland, placenta, prostate (stroma), ovary (theca cells), testes (Leydig cells), epididymis, bone, breast, various regions of the brain, liver, central nervous system, cardiovascular system and white adipose tissue (Nilsson *et al.,* 2001).

ERβ has a broader tissue distribution and is expressed in, for example, colon, prostate (epithelium), testis, ovary (granulosa cells), bone marrow, salivary gland, vascular endothelium, pineal gland, thyroid gland, parathyroids, adrenals, pancreas, gallbladder, skin, urinary tract, lymphoid, erythroid tissues, certain ERα-lacking brain regions and muscle (Ciocca and Roig, 1995; Gustafsson, 1999).

In teleost fish, a third form of ER subtype (ERβ2 or ERγ) has been reported, first identified in a species of marine ray-finned fish, the Atlantic croaker (*Micropogonias undulates* (Hawkins *et al.,* 2000; Menuet *et al.,* 2002). Unlike ERα - which is homologous across vertebrates - or ERβ - which shares the greatest identity with mammalian ERβ - phylogenetic analysis shows that ERβ2 has no mammalian homologue, has several key amino acid changes and is likely to have arisen through gene duplication from ERβ, early in the teleost lineage (Hawkins and Thomas, 2004). Equally, a second form of ERα (ERα2) has since been described in the Rainbow trout (*Oncorhynchus mykiss*) which is also thought to exist in other teleost species (Nagler *et al.,* 2007). In general, teleost ERs seem to bind 17β-estradiol (E2) with less affinity than mammals - approximately 3 to 5-fold less for the rainbow trout ERa compared to human - (Petit *et al.,* 1995). Whilst both ERα and ERβ bind to E2 with similar affinity in mammals (Paech *et al.,* 1997) in teleosts, ERβ forms appear to have a higher affinity. This characteristic has been described in several species including the Atlantic croaker, zebrafish (*Danio rerio*) and channel catfish (*Ictalurus punctatus*) (Xia *et al.,* 2000, 1999; Menuet *et al.,* 2002; Hawkins and Thomas, 2004).

As with other vertebrates, many studies have reported disparate tissue expression patterns of the different ER subtypes and their genes in fish. In general, both ERα and ERβ1 are concentrated in the gonad and liver (Tchoudakova *et al.,* 1999; Ma *et al*., 2000; Socorro *et al.,* 2000; Menuet *et al.,* 2002; Filby and Tyler, 2005), which is consistent with the critical role of oestrogens in gonadal sex differentiation and development and in the hepatic production of vitellogenin (VTG), a precursor protein to egg yolk, and vitelline envelope proteins (VEPs) required for oocyte synthesis (Devlin and Nagahama, 2002). In particular, the close correlation between the up-regulation of hepatic ERα and VTG induction reported in a number of teleost species, strongly suggests that this particular ER plays a more dominant role in regulating vitellogenesis (Menuet *et al.,* 2004**;** Sabo-Attwood *et al.,* 2004; Filby and Tyler, 2005). Furthermore, one or the other of the distinct ER subtypes can predominate in different tissues, e.g. the liver in post spawning female roach has been shown to contain higher ERα levels than in the ovary where ERβ1 appears to be more highly expressed (Menuet *et al.,* 2001) whilst in goldfish (*Carassius auratus*) higher expression levels of ERα have been observed in the pituitary and ovary, with greater transcript levels of ERβ1 found in the testis (Choi and Habibi, 2003). In contrast, a 2004 study using European sea bass (*Dicentrarchus labrax*) by Halm *et al.,* suggests that the ER subtype ERβ2 is predominantly expressed in somatic tissues, with similar findings reported in fathead minnow by Filby and Tyler (2005). Tissue expression of the different ER subtypes has also been shown to differ between species. For example, in sea bream, ERα expression has been reported to occur mainly in the testis, liver and heart, while ERβ1 is found in most tissues, being more abundant in ovary, testis, liver, intestine and kidney (Pinto *et al.,* 2006). In contrast, tissue distribution patterns of ERα, ERβ1 and ERβ2 in zebrafish appears to occur predominantly in the brain, pituitary, liver and gonad (Menuet *et al.,* 2002; Tingaud-Sequeira *et al.,* 2004; Froehlicher *et al.,* 2009; Gorelick *et al.,* 2014). Several studies have also elucidated that specific developmental processes are signalled through the different ER types (and sub-types). In the zebrafish for example, knockdown of ERβ2, but not ERα, suppresses neuromast cell development in the brain (Froehlicer *et al.,* 2009).

1.6.3 Evidence for health impacts of endocrine disruption in aquatic wildlife

The first indication of the potential endocrine disrupting health effects of WwTW effluents on the aquatic environment was revealed by studies in the UK on sexual disruption in fish after observations from anglers of the occurrence of intersex fish (in this instance wild roach, *Rutilus rutilus*) living downstream of a WwTW and in an effluent settlement lagoon (Sweeting, 1981). These initial reports led to an impetus of scientific research with subsequent findings of vitellogenin (VTG), normally expressed in high concentrations in spawning females only, in the plasma of male rainbow trout in a location near a WwTW (Van Bohemen *et al.,* 1982). With the establishment that VTG is produced under the control of oestrogen (Scott and Sumpter 1987) further controlled studies using caged rainbow trout placed upstream and downstream of WwTW, using VTG as a biomarker of oestrogen exposure, produced strong evidence of a causal link with the estrogenic activity of effluent discharges and feminisation in fish (Purdom *et al.,* 1994; Harries *et al.,* 1996, 1997), a phenomenon which has subsequently been reported in other studies across the world including the USA, Japan, Germany and Switzerland (Herman and Kincaid, 1988; Jobling and Sumpter, 1993; Bortone and Davis, 1994; Sumpter, 1995; Desbrow *et al.*, 1998; Gibbons *et al.*, 1998; Tyler *et al.*, 1998; Larsson *et al.*, 1999; Sole *et al.*, 2000; Karels *et al.*,2001). This in turn has been shown to be largely attributable to the presence of EDCs found in treated wastewater effluent, primarily oestrogenic substances such as 17β-estradiol (E_2) and 17α-ethinylestradiol (EE_2) and/or weaker oestrogen mimics such as NP (Routledge *et al.*, 1998; Kidd *et al.,* 2007).

Since the initial discovery of feminised roach in UK rivers, several studies have confirmed that the occurrence of sexual disruption in fish is neither species or habitat specific, with subsequent reports of intersex fish in other wild freshwater species such as the gudgeon (*Gobio gobio*) (van Aerle *et al.,* 2001), common bream (*Abramis brama*), common carp (*Cyprinus carpio*) and shovelnose sturgeon (*Scarphirhynchus platyorhynchus*) (Harshbarger *et al.,* 2000) and the appearance of feminised fish in estuarine species such as the European and Japanese flounders (*Platichthys flesus* and *Pleuronectes yokohamae*, respectively) (Lye *et al.,* 1997; Allen *et al.,* 1999; Hashimoto *et al.,* 2000; Kirby *et al.,* 2004). However, most

research into the health impacts of EDCs in fish has been largely limited to laboratory-based exposure studies using a handful of model fish species, despite further evidence of the variability in sensitivity of different fish species to a wide variety of chemicals (Van den Belt *et al.* 2003; DeForest *et al.* 2012; Lange *et al.* 2012). This is important when trying to extrapolate data to native fish populations in the wild as it brings into question the suitability of these models as indicators or predictors of potential health effects and population level impacts, particularly considering that fish exhibit greater phylogenetic diversity than any other group of vertebrates (Fishbase, 2018).

A number of EDCs frequently detected in WwTW effluents such as pesticides, phthalates, phenols and pharmaceuticals (Sohoni and Sumpter, 1998; Svanfelt *et al.,* , 2010) have also be shown to directly interfere with thyroid receptors (Zoeller, 2005) and thus potentially disrupt normal thyroid function (Brucker-Davies, 1998, Li *et al.,* 2008) as seen in herring gulls (*larus argentatus*) Moccia *et al.,* 1986) and salmon (Moccia *et al.,* 1981). In addition, there is some evidence of neurological level effects, with exposure to certain polychlorinated biphenyls (PCBs) shown to disrupt the function of gonadotropin-releasing hormone (GnRH) containing neurons (Gore *et al.*, 2002).

A wide range of EDCs directly related to WwTW effluent exposures, such as heavy metals, alkylphenols, pesticides, pharmaceuticals and xenoestrogens have been shown to bio-accumulate/concentrate in the fatty tissue of aquatic organisms. In the case of bio-accumulation, this occurs when the chemical concentration within an organism achieves a level that exceeds that of its environment as a result of chemical uptake through dietary ingestion (trophic transfer), dermal adsorption or transport across the respiratory surface; with bio-concentration this occurs through the respiratory surface and/or the skin only (Geyer *et al.,* 2000). A number of studies have shown that EDCs, mainly estrogenic chemicals such as E_1, E_2, EE_2 and BPA, and some waterborne alkylphenols, i.e. NP, can be detected at high concentrations in the bile of fish exposed to effluents (Fenlon *et al.,* 2010; Da Silva *et al.,* 2013; Yang *et al.,* 2014). In addition, an indication of the potential for EDCs to bio-magnify (i.e. the process whereby a chemical is passed upwards through the food chain to higher trophic levels, such that it exceeds the expected concentration where

equilibrium occurs between an organism and its environment) has also been reported in numerous studies (Fisk *et al.,* 2005; Vernouillet *et al.,* 2010; Ruhí *et al.,* 2016). As such, species at higher trophic levels, particularly top predators, are more likely to be at greater risk in comparison with those at lower levels. However, other studies such as those conducted by Vine *et al.,* (2005) into sexual disruption in the European pike (*Esox Lucius*), a top predatory fish living in English rivers, and by Du Bowen *et al.,* (2014) on the trophic dilution of human pharmaceuticals across trophic positions in an effluent dependent stream, did not find any evidence of the bio-magnification of EDCs, through a predator-prey relationship.

1.6.3 Health impacts of endocrine disruption in humans

A large number of studies have linked exposure to EDCs with a wide range of physiological effects in humans, including developmental disorders (Mouritsen *et al.,* 2010), cancer (e.g. breast cancer, prostate cancer (Safe and Papineni, 2006; Prins *et al.,* 2007, Pisapia *et al.,* 2010) diabetes and other metabolic syndromes (Lee *et al.,* 2007), as well as reproductive disorders such as infertility and sub-fertility (Ma, 2009; Jeng, 2014).

One of the best-studied examples of the adverse effects of EDCs on human health is *inutero* exposure to diethylstilbestrol (DES), a potent synthetic nonsteroidal oestrogen given to pregnant women from the 1940s to 1970s to help prevent miscarriage. Its use was subsequently discontinued when it was shown that exposed female offspring had an increased risk for multiple birth defects (including structural reproductive tract anomalies), certain cancers (e.g. early-onset clear cell adenocarcinoma of the vagina, uterus and cervix) and other diseases, such as a higher risk of infertility and more complicated and unsuccessful pregnancies (Troisi *et al.,* 2007; 2013). The harmful effects of DES exposure have also been reported in exposed male offspring, with elevated rates of urogenital malformations, cryptorchidism (undescended testes), testicular cancer, low sperm density and mobility (Gill *et al.,* 1976, Palmer *et al.,* 2005). Subsequently, a plethora of examples is emerging for evidence of the epigenetic effects of EDCs as a result of environmental exposures during critical periods of growth and development. These

include EDCs such as BPA, phthalates, PCBs, pesticides, dioxins, and tributyltin (TBT), among others. (Wolf *et al.,* 2008; Lin *et al.,* 2010; Newbold & Heindel, 2010).

Epigenetic effects are heritable changes in gene expression that do not involve changes to the underlying DNA sequence, i.e. a change in phenotype without a change in genotype (Rozek *et al.,* 2014).

In addition, there is mounting evidence in both human and animal models, of the ability of EDCs to induce changes to germ cells – precursors to egg and sperm cells – potentially making their effects heritable not just to direct offspring but to subsequent generations. For example, exposure to plasticizers (DEHP, MEHP) results in the premature loss of germ cells and downregulation of Leydig cells (Zhang *et al.,* 2008; Yao *et al.,* 2010; Erkekoglu *et al.,* 2012), whilst the process of spermatogenesis is disrupted by estrogenic compounds (BPA) and organophosphates (TOCP, SCOTP), (Aoki and Takada, 2012, Chen *et al.,* 2012).

Growing evidence also suggests a causal link between pre-natal or early life exposure to certain EDCs and obesity in humans and animal models (Sargis *et al.,* 2010; Ariemma *et al.,* 2016). These chemicals have since been termed ‘‘obesogens,’’ based on the idea that they can downregulate lipid metabolism and adipogenesis (Bertoli, *et al.,* 2015). In particular, the weak environmental oestrogen, BPA has been shown to act as an obesogen, with several studies reporting a positive association between obesity and higher urinary BPA concentrations in adults and children (Trasande *et al.,* 2012; Wang *et al.,* 2012; Li *et al.,* 2013). In addition, in vitro studies have indicated the disruptive effects of BPA on adipocyte development as well as on the homeostatic control of adipogenesis and early energy balance (Grun *et al.,* 2006). A comprehensive review of the evidence for endocrine disruption in both animal models and human clinical observations can be found in Gore *et al.,* 2015.

1.7 Environmental oestrogens

It is now well established globally that one of the main characteristics of WwTW effluent that often impacts receiving waterways is its oestrogenicity. Even in highly developed countries such as the UK and despite advances in wastewater treatment

and a general reduction in the incidence of gross point source pollution, effluent from most WwTW is oestrogenic, with around a third of English rivers currently thought to contain levels of oestrogenic chemicals sufficient to cause some endocrine disruption in fish (Williams *et al.,* 2009)**.** This is highly variable however and is dependent on a number of factors including water quality and seasonal changes. For example, a study on the levels of E_1 and E_2 in composite effluent samples taken on consecutive days saw a 2 to 3-fold difference in concentrations (Williams *et al.,* 2009).

In general, steroidal oestrogens are some of the most potent EDCs found in WwTW effluents, with many studies confirming their ability to significantly affect fundamental biological processes, such as sexual development and reproduction, most commonly reported in freshwater fish. These disruptions can present themselves in a number of different ways: a sex ratio biased in favour of females; occurrence of intersex individuals (having simultaneous presence of ovarian and testicular tissue in the same gonad); delayed sexual maturation; reduction in gonadosomatic index (GSI); histological alterations in the gonads; disruption of spermatogenesis; decrease in fertilization rate; alterations in circulating steroid concentrations; altered biomolecules; alterations in reproductive behaviour (reviewed by Milnes *et al.,* 2006).

Four oestrogens commonly found in WwTw effluents include three natural steroids, oestrone (E_1), 17β-oestradiol (E_2) and oestriol (E_3) and one synthetic compound 17α-ethinylestradiol (EE_2). In addition, the presence of various xenoestrogens (or false oestrogens) such as nonylphenol and BPA, are frequently detected in sewage effluent. In the UK, levels of reported concentrations of E_1 (the most common steroid oestrogen found in WwTW effluents (Katsu *et al.,* 2007) with levels of between 1 and 88 ng/L for E_2, and 1 to 76 ng/L for E_3, (Desbrow *et al.,* 1998; Rodgers-Gray *et al.,* 2001).

Due to its persistent nature and widespread occurrence, one of the most recalcitrant estrogens found in the aquatic environment is EE_2, commonly used as an active ingredient in oral contraceptives or in hormone replacement therapy. Worldwide, it is estimated that more than 100 million women of a reproductive age use the contraceptive pill (UN, 2011) with an average EE_2 uptake of around 20-35 μg per day

(BKH, 2001). Whilst it is known that EE_2 enters WwTW primarily in a conjugated, inactive form, i.e. as sulfuric and glucuronic acids (Larsen *et al.,* 2008) once broken down it can be bio-transformed back into an active form with a full oestrogenic potency 10 to 50 times higher than that of other natural oestrogens (Segner *et al.,* 2003). Reported concentrations of EE_2 in WwTW effluents range from 0.1 to 9 ng/L with levels in UK freshwater systems known to reach 3.4 ng/L (Williams *et al*., 2003).

This is particularly concerning as several studies have shown that environmentally relevant concentrations of EE_2 can elicit significant endocrine disrupting effects. For example, work by Fenske *et al*., (2001, 2005) with zebrafish (*Danio rerio*) revealed increases in vitellogenin synthesis and disruptions in reproductive development in males in exposures of 1.67 ng l^1 and 3 ng l^1 respectively, comparable with the effects seen in studies in early life work on roach and fathead minnow for similar exposure regimes (Lange *et al.,* 2009). In addition, a study by Santos *et al*. demonstrated that current environmental concentrations of EE_2 can also decrease the quality and quantity of gametes produced by both male and female zebrafish (Santos *et al.,* 2007). Conversely, fewer studies have been conducted into the effects of exposure to WwTW effluents themselves or into environmentally relevant concentrations of oestrogens.

1.7.1 Experimental methods for assessing oestrogenic activity and the effects of environmental oestrogens in wastewater effluents

In general, there are three main ways to assess the oestrogenic activity/determine the effects of oestrogenic chemicals found in wastewater effluent; chemical analyses, *in vitro* assays and *in vivo* assays.

A number of *in vitro* assays have been developed to assess the potential oestrogenic activity of single compounds or complex mixtures such as effluents, without the need for chemical identification. These tests are generally performed with individual cells or molecules to assess dose-response relationships. *In vitro* approaches are sensitive, specific and often cheaper and more rapid than *in vivo* methods. Moreover, they offer ethical advantages (with respect to the use of less test animals) and also tend to generate less waste (Baksi and Frazier, 1990). However, one of the main drawbacks of *in vitro* systems is they do not account for the metabolism of a

chemical or make allowances for bio-concentration - which can serve to enhance the biological effect of an EDC - thus limiting further inference to effects that might occur *in vivo* (Zacharewski, 1997; Folmar *et al.,* 2002; Murk *et al.,* 2002).

There are four main categories of *in vitro* bioassays available, each providing a measure of oestrogenic activity dependent on which endpoint of the biological response to natural estrogens they measure; 1) oestrogen receptor competitive binding assays, 2) reporter gene assays, 3) gene expression assays, and 4) cell proliferation assays. Oestrogen receptor competitive binding assays, such as the enzyme-linked receptor assay (ELRA), measure the ability of the chemical of interest to compete with oestrogen for binding to an ER (Seifert, 2004). They are relatively simple to run and amenable to high-throughput screening (due to their low cost and speed) but are unable to differentiate between agonistic and antagonistic EDCs and are generally poor predictors of whole-organism effects.

An alternative to this system is the reporter gene assay, which measures oestrogenic activity through receptor-mediated gene induction, by quantifying the production of a specific oestrogen responsive protein/enzyme after incubation. Reporter gene assays are generally carried out with genetically engineered yeast or mammalian cells transfected with an ERE DNA sequence linked to a reporter gene such as luciferase or galactosidase (Zacharewski, 1997; Kinnberg, 2003). An example of both a yeast cell based and mammalian cell reporter gene assay (and two of the most commonly applied reporter gene assays) are the yeast estrogen screen (YES) and the ER-mediated, luciferase reporter gene-expression (ER- CALUX) assay (Routledge and Sumpter, 1996; Legler *et al.,* 1999; De Boever *et al.,* 2001). In general, yeast-based reporter gene assays are more robust than mammalian-based assays but less sensitive and are comparable to estrogen receptor competitive binding assays, in such that they are poor predictors of whole-organism effects. However, unlike mammalian-based reporter gene assays they do not require a high level of expertise and specialized laboratory facilities and are therefore relatively inexpensive to run.

In addition, gene expression assays are widely used *in vitro* to provide a measure of a natural cellular response to oestrogenic stimulation; as some cells naturally express oestrogen-responsive proteins (such as VTG in fish hepatocytes (Petit *et al.,*

1997; Tremblay and Van Der Kraak, 1998) or pS2 protein in breast cancer cells (Rosenberg and *et al.,* 1999), this endogenous gene expression can subsequently be used to detect oestrogenic activity in a sample. A major advantage of gene expression assays is that they are able to detect receptor, non-receptor, and non-genomic effects. However, as induction of oestrogen-responsive proteins can also arise via non-ER mediated pathways (Zacharewski, 1997) any observed responses may not always be specific to oestrogenic activity, leading to a potential for false positives. In addition, any results are also often cell-specific and may also not be extrapolated to other tissues or species.

A final example of an *in vitro* bioassay is the cell proliferation assay, which utilises cell lines which are oestrogen-dependent for growth, e.g. human breast cancer cells – (Byford *et al.,* 2002; Charles and Darbre, 2009). As with gene expression assays, cell proliferation assays can also measure several oestrogenic pathways at once, but are not always specific to oestrogenic activity, again potentially leading to false positive results.

1.7.1.1 In vivo assays

In vivo assays work on the principal of measurable physiological, morphological or behavioural responses - which are known to be induced following exposure to oestrogenic chemicals. Whilst *in vivo* assays are more costly and time-consuming than *in vitro* bioassays, they do incorporate important biological processes that are limited in the latter, such as metabolic biotransformation and active transport mechanisms. Moreover, while *in vitro* bioassays are largely limited to ER-mediated effects, *in vivo* assays can also assess oestrogenic effects through non-ER-mediated pathways (e.g. interference with steroid and receptor biosynthesis). However, due to the complex feedback mechanisms involved in endocrine communication, any observed oestrogenic effect in many *in vivo* bioassays may also be related to anti-androgenic effects and is often impossible to distinguish between the two.

In fish, several endpoints can be used as *in vivo* bioassays for measuring oestrogenic responses to EDCs. One of the most widely used and extensively documented biomarkers is VTG induction (Denslow *et al.,* 1999; Craft *et al.,* 2004; Bogers *et al.,* 2006; Cosnefroy *et al.,* 2009) which offers a sensitive, rapid, dynamic range (up to a million-fold induction) and high specificity for oestrogens (Thomas-

Jones *et al.,* 2003). Levels of VTG protein in the plasma can be quantified using a variety of methods, such as radioimmunoassays (Purdom *et al.,* 1994; Routledge *et al.,* 1998) immunoblotting (Petrovic *et al.,* 2002) and the enzyme-linked immunosorbent assay (ELISA) (Denslow *et al.,* 1999). ELISA are possibly the most commonly used method to measure VTG induction and have been developed for use in many different species of fish, including Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), and fathead minnows (*Pimephales promelas*) (Korsgaard and Pedersen, 1998; Lomax *et al.,* 1998; Sherry *et al.,* 1999; Folmar *et al.,* 2000; Holbech *et al.,* 2001; Pait and Nelson, 2003; Ataria *et al.,* 2004; Nilsen *et al.,* 2004). VTG induction can also be measured by quantifying VTG mRNA levels in the liver or gonads or in whole body homogenates using methods such as hybridization protection assay (HPA) (Thomas-Jones *et al.,* 2003), real time reverse transcription polymerase chain reaction (real time RT-qPCR) (Leusch *et al.,* 2005) and microarrays (Bowman and Denslow,1999; Lattier *et al.,* 2001; Islinger *et al.,* 2002; Alberti *et al.,* 2005; Denslow *et al.,* 2007). Induction of ER genes (e.g. liver mRNA and cellular ER) can also be used to determine exposure to oestrogenic chemicals in fish, using similar techniques as described above (Denslow *et al.,* 2001b; Larkin *et al.,* 2002; Leusch *et al.,* 2005).

Other indicators of exposure to chemicals with oestrogenic activity include morphological endpoints such as ovo-testis (the presence of both oocytes and testicular tissue in the gonads of the same individual (Bortone and Davis, 1994) , increased liver mass - measured by the hepatosomatic index (HIS) – and relative gonad size - measured by the gonadosomatic index (GSI) (Sindhe and Kulkarni, 2004; Yang and Baumann, 2006) and a reduction/abnormal development of secondary sexual characteristics (i.e. tubercles and fatpads in male fathead minnow, (Pawlowski *et al.,* 2004; Filby *et al.,* 2007). In addition, changes in normal behavioural responses can also be an indicator of oestrogenic exposure (e.g. a reduction in sexual activity such as the frequency of copulatory attempts of male fish, disruption in nest building and diminished courting responses in females (Doyle and Lim, 2002; Brian *et al.,* 2006; Coe *et al.,* 2010).

1.7.1.2 Transgenic Fish

The potential for transgenic fish was first realized when it was shown that plasmid

DNA injected into the cytoplasm of fertilized eggs could integrate into the genome and be stably transmitted to successive generations (Zhu *et al.,* 1985; Stuart *et al.,* 1988). Subsequently, there has been a rapid growth in the use of transgenic fish lines in various fields of scientific research, such as studies to inform on genetic and developmental processes, and as model biosensor systems for chemical screening in ecotoxicological research, where they can inform on tissue targets and effect mechanisms (Blechinger *et al.,* 2002; Bogers *et al.,* 2006; Gorelick and Halpern 2011; Lee *et al.,* 2012; Moro *et al.,* 2012; Nguyen *et al.,* 2012). In addition, transgenic research has been incorporated into studies of physiological processes in fish species such as growth rate, cold tolerance and disease resistance (Wang *et al.,* 1995).

Transgenic fish offer the advantages of both *in vivo* and *in vitro* systems such as rapid throughput analysis, sensitivity and moderately cheap set up costs (establishing a TG line, however, is expensive and fairly time consuming). There are several different methods which can be used to produce transgenic fish but all require a transgenic construct (created using recombinant DNA techniques) with a promoter and a gene. Once the construct with the gene of interest has been produced it is subsequently transferred into the cytoplasm of fertilized fish eggs at the one cell stage - most commonly using microinjection or electroporation - and the fish grown to adulthood. The presence of the transgene is then established through Southern blotting and/or polymerase chain reaction (PCR) on genomic DNA generally isolated from external fin tissue. Single founder (F0) fish are then crossed with single wild-type (non-transgenic) fish and their off spring subsequently crossed with each other to verify germ-line integration and to establish a homozygous transgenic line (Zhu *et al.,* 1985; Stuart *et al.,* 1988).

Transgenic fish work on the principle that certain genes - typically enzymes or receptors – are responsive to certain chemical pollutants therefore exposure of the fish to the chemical(s) of concern generates the activation of inducible promoter elements, that in turn triggers expression of the reporter gene, which is generally positioned downstream of the promoter of the inserted transgene. Whilst various reporter genes have been used in TG fish research – such as *E. coli b* -galactosidase (lacZ), chloramphenicol acetyltransferase (CAT), neomycin

phosphotransferase (NPT), Kaede, yellow fluorescent protein (YFP), DsRed and mCherry (Culp *et al.,* 1991; Ando *et al.,* 2002; Nagai *et al*. 2002; Uzbekova *et al.,* 2003) - the most commonly used to date are luciferase (LUC) and green fluorescent protein (GFP).

LUC originates from the firefly *Photinus pyralis* and encodes the enzyme luciferase, which causes the cell that expresses it to catalyse luciferins and produce light (Contag and Bachmann, 2002) hence allowing visualisation of the expression of the target gene of interest and associated cellular and physiological processes in live animals. As it is highly sensitive, quick and simple to use, LUC is commonly used in cell-base reporter assays and for the quantitative measurement of gene expression. In addition, as light excitation is not needed for luciferase bioluminescence there is nominal auto-fluorescence and therefore virtually no interference from background fluorescence (Williams *et al.,* 1989).

However, as a method LUC requires a costly substrate and is fairly unstable, i.e. LUC half-life varies from 1.5 to 3 hours, dependent on the host cell and the gene construct (Goodman and Gao, 1999; Leclerc *et al.,* 2000). Several research groups have shown that luciferase can be detected in live zebrafish embryos, larvae and adults (Weger *et al.,* 2012; Chen *et al.,* 2013,).

GFP is a fluorescent protein, first isolated from the bioluminescent jellyfish *Aequoria Victoria*, which exhibits a bright green fluorescence via microscopy. It can be visualised directly in living cells/tissues and in whole organisms and responses can be quantified in real time. Furthermore, GFP can be detected in zebrafish during both embryonic and larval development, and when used in conjunction with zebrafish mutant lines that lack skin pigmentation, in later life stages (Cooper *et al.,* in press). The advantages of GFP over LUC include superior intensity, inherent fluorescence and comparatively high photo-stability.

Since the development of the first GFP transgenic fish in 1995 (Amsterdam *et al.,* 1995) GFP transgenic fish technology has been widely used in many areas such as analyses of gene expression patterns, tissue/organ development, analysis of tissue-specific promoters/enhancers, cell migration and mutagenesis screening (Driever *et*

al., 1996; Haffter *et al.,* 1996; Gong *et al.,* 2001; Udvadia and Linney 2003). Its' application in the development of biosensor transgenic zebrafish to monitor EDCs is also widely recognised (Blechinger *et al.,* 2002; Tong *et al.,* 2009; Chen *et al.,* 2010; Gorelick and Halpern 2011; Lee *et al.,* 2012a,). With both LUC and GFP reporter genes it is possible to develop a more integrative analysis and understanding on the uptake, distribution, accumulation and action of the chemicals in the tissue of live fish.

1.7.1.3 Examples of transgenic fish models

Whilst there are a large number of available transgenic fish lines (spanning a number of divergent species) including the channel catfish *Ictalurus punctatus* (Dunham *et al.,* 2002b), common carp *Cyprinus carpio* (Dunham *et al.,* 2002a), goldfish *Carassius auratus* (Wang *et al.,* 1995) mud loach *Misgurnus anguillicaudtis* (Nam *et al.,* 2001), rainbow trout *Oncorhynchus mykiss* (Uzbekova *et al.,* 2000), Atlantic salmon *Salmo salar* (Saunders *et al.,* 1998) and the coho salmon *Oncorhynchus kisutch*, (Devlin *et al.,* 2004), two of the most widely used species for producing transgenic fish lines are the medaka (*Oryzias latipes*) a small freshwater teleost fish native to east and mainland southeast Asia and the zebrafish (*Danio rerio*), again a small freshwater teleost, originating from the rivers of northern and eastern India.

The popularity of these species as transgenic models is largely due to the fact that both are easily bred/maintained, have high fecundity rates, produce large numbers of transparent eggs (allowing for clear observation of developmental processes) and possess fully sequenced genomes offering extensive genetic resources. For zebrafish, the presence of a comparatively soft chorion also means that microinjection of DNA and genetic constructs (the most popular method for transferring transgenes into a fertilized egg) is considerably easier than with many other fish species. In addition, orthologues of most human genes can be found in the zebrafish (approximately 70% identical in terms of their amino acid residue sequence) making them ideal for modelling human disease (Blader & Strahle, 2000). Whilst high quality genomic resources are also available for the medaka (Matsumoto *et al.,* 2009), it has a much smaller genome - around half the size of zebrafish and one-third that of humans (Naruse *et al.,* 2004).

Several transgenic medaka and zebrafish lines have been developed to study the potential health impacts of exposure to various chemical pollutants (including EDCs) and other environmental stressors such as oxidative stress, increased temperature and heavy metals (Mattingly *et al.,* 2001; Blechinger *et al.,* 2002; Ji *et al.,* 2012). In particular, transgenic fish lines have been specifically created for assessing health effects of oestrogenic chemicals. These include lines with an oestrogen responsible promoter derived from vitellogenin or choriogenin genes, the latter being a precursor protein of the inner layer of the egg envelope which is similarly synthesized in the liver of maturing female fish in response to oestrogens (Zeng *et al.,* 2005; Bogers *et al.,* 2006; Kurauchi *et al.,* 2008; Salam *et al.,* 2008; Chen *et al.,* 2010; Petersen *et al.,* 2013). Whilst these systems can be used for screening chemicals for oestrogenic activity *in vivo* in real time - which can subsequently be applied to inform on cumulative responses to oestrogens - they are limited for use in environmental monitoring as they lack high sensitivity, with responses restricted to the liver. In contrast, a number of transgenic lines developed in zebrafish for detecting oestrogens employing oestrogen response elements (ERE), using either luciferase or GFP as reporter genes, have demonstrated detection levels of environmental oestrogens down to 1 ng/L, as well as tissue specific expression, and have also shown that responses to oestrogenic EDCs can differ between different developmental life stages (Bogers *et al.,* 2006; Lee *et al.,* 2012a; Gorelick *et al.,* 2014) The first of these transgenic biosensor zebrafish (developed by Legler *et al.,* 2000) incorporated an oestrogen responsive LUC reporter gene, regulated by three EREs upstream of a TATA minimal promoter. In this model, juvenile fish (35 dpf) were shown to be responsive to E_2 at concentrations down to 0.1 nM (27.2 ng/L) (for a 96-hour exposure) with the testis being the most responsive target tissue.

1.8 Generation of the ERE-TG Zebrafish model

The ERE-TG fish used in this thesis were developed at the University of Exeter, using a Gal4ff-UAS (Upstream Activation sequence)/GFP signalling system, not previously applied in a transgenic fish biosensor system, mediated by a *Tol2* transposon system, incorporating three tandem repeats of oestrogen response elements (3ERE) capable of binding to all three ER subtypes and Gal4ff.

The *Tol2* transposon is a popular choice in the development of transgenic fish due to

the fact that it has been found to reduce mosaicism and enhance the germ-line transmission of transgenes (Hermanson *et al.,* 2004). Gal4ff is a modified form of the yeast transcription factor Gal4 providing high transcriptional activation with low toxicity, which binds its target sequence UAS to activate gene transcription, driving the expression of the second reporter gene, EGFP (Enhanced Green Fluorescent Protein). This method was employed to generate a two-step amplification of the estrogenic signal, in order to produce a more sensitive response system for detecting exposure to oestrogens (**Fig 3**).

Fig. 3. 3ERE-Gal4ff/UAS-GFP system in zebrafish. The ERE-Gal4ff transgene contains three synthetic EREs, one TATA, a Gal4ff reporter, and two *Tol2* elements; and the UAS-GFP transgene contains five tandem repeats of the UAS-Gal4 binding sequence, an E1B minimal promoter, and an EGFP reporter. An oestrogenic signal is detected when the chemical–ER complex binds to the ERE, which activates Gal4ff; subsequently, Gal4ff protein binds to UAS to induce EGFP (Source: Lee et al., 2012).

Preliminary studies with the ERE-TG zebrafish model have shown it to be a highly sensitive model system, capable of responding to a wide range of oestrogens that are diverse in nature (spanning steroidal oestrogens to an alkylphenol [NP] and a plasticizer [BPA]) at environmentally relevant concentrations, with reported response sensitivities of 1 ng/L (EE_2), 5 ng/L (E_2), 100 ug/L (BPA) and 1ug/L (NP). In addition, and unlike some previous transgenic fish lines where tissue responses have been restricted to the liver, the transgenic fish have shown multiple tissue responses for oestrogenic (GFP) signaling for steroid oestrogen exposure including the liver, heart, brain, jaw, otic vesicle, skeletal muscle (somite and cranial), ear/eye ganglions, lens and neuromasts .This is consistent with the known expression patterns (collectively) for the different ER subtypes and some other ERRs in fish, mammals and humans, and linked with the very diverse roles of oestrogens in vertebrate function (Heldring *et al.,* 2007). Tissue-specific expression patterns for the different environmental oestrogens indicating differences in tissue toxicities have also been observed (Lee *et*

al., 2012). As such, the ERE-TG zebrafish model could be very usefully applied to study how real-world mixtures (e.g. estrogenic WwTW effluents) affect developmental process and fish health.

1.9 Aims of this study

The overall aims of the work presented in this thesis were to (i) investigate the potential for health impacts of environmental oestrogens, including complex mixtures contained within effluents from WwTws, using an ERE-GFP-TG zebrafish as an experimental model; (ii) advance the model for use in environmentally relevant toxicological research and chemical testing and screening; (iii) establish how fluorescence responses in the transgenic zebrafish model compared in terms of sensitivity for the detection of oestrogens with vitellogenin (VTG) induction, a well-established biomarker for oestrogen exposure in fish.

An integrative approach, using a wide range of techniques was applied to better establish exposure, target tissues and biological effects of environmental oestrogens, including embryology, qPCR for target gene expression analysis, histopathology, LC-MS, in life *in vivo* exposures and fluorescence imaging.

In order to achieve the above aims, the following experimental work was conducted (presented in the form of scientific papers that will be submitted for publication in peer-reviewed journals), with the following specific objectives:

Objective 1 (Chapters 2 and 3) - To evaluate the sensitivity and repeatability of the ERE-GFP zebrafish in assessing the biological potencies and effects of exposure to an oestrogenic wastewater treatment work (WwTW) effluent.

In order to achieve this aim, in the first part of this work (Chapter 2), ERE-GFP zebrafish embryos (*n*= 50 per treatment x 3 replicates) were exposed from fertilisation to 4 days post-fertilisation to either a dilution water control (DWC), a reference oestrogen positive control (EE_2 10 ng/l) or either a 100% or 50% effluent concentration from three different WwTW effluents (Exeter, Plymouth and Tiverton WwTWs), to investigate for responding target tissues and potential health outcomes

and to compare how these responses varied both seasonally and between treatment works. All exposures were repeated every three months over a 12-month period.

In the second part of this work (Chapter 3) a new oestrogen transgenic zebrafish strain, ERE-GFP-Casper, was applied to compare for tissue responses and sensitivities and for potential health effects following exposures to a single WwTW effluent. The model was generated using the aforementioned ERE-GFP zebrafish, crossed with a Casper line (Green *at al.*, 2016). The translucent nature of this model allowed for an improved fluorescence detection and quantification in specific tissues and in later developmental life stages. Using a purpose-built facility at the University of Exeter, ERE-GFP-Casper zebrafish (*n* = 180 per treatment x 3 replicates) were exposed to either a DWC, a reference oestrogen positive control (EE_2 10 ng/L) or a wastewater effluent derived from Countess-Wear (Exeter) WwTW at a concentration of either 100% or 50% for the following developmental life periods: (i) 0-90 days (ii) 21-60 days (iii) 60-90 days.

A final element of both chapters sought to establish how GFP responses in the ERE-GFP zebrafish models compared in terms of sensitivity for the detection of oestrogens with VTG induction (quantified through direct comparison of *gfp* with *vtg* mRNA expression using RT-qPCR)

Objective 2 – (Chapter 4) To assess the sensitivity and repeatability of the ERE-GFP zebrafish system for determining whole body responses and characterising the tissue specificities of oestrogenic chemicals to inform on target tissues and potential health outcomes.

In order to achieve this aim ERE-GFP zebrafish embryos (*n*= 50 per treatment x 3 replicates) were exposed from fertilisation to 4 days post-fertilisation to either a dilution water control (DWC), or a suspected environmental oestrogen and responses measured via VTG production and GFP expression. The work also sought to provide information on dose and time-related responses to oestrogens across the different body tissues, to identify both the unique tissue specific expression patterns and thresholds for responses of each of the different oestrogens

and to help distinguish commonly susceptible tissues that appeared sensitive to all chemicals.

Objective 3 (Chapter 5) - To establish tissue patterns of low-level endogenous oestrogen expression in the normal development of the zebrafish and determine whether these differences could be used to distinguish the sex of individuals.

To achieve this aim ERE-GFP-Casper zebrafish, in the absence of chemical exposure, were observed at several time points during their ontogeny (i.e. 5, 10, 20, 30, 40, 50 and 60 dpf) using fluorescence microscopy to measure GFP expression. Those expressing GFP were separated from those that did not express GFP into individual tanks and grown to 60 dpf when they were subsequently sacrificed and analysed via gonadal histopathology to identify their sex.

Chapter 6: General Discussion

This chapter places this research within the field, providing a critical overview of the major findings from this thesis work. The limitations and challenges of the research are discussed along with recommendations for possible future work.

Chapter 2

An integrative approach using an ERE-GFP transgenic zebrafish model to assess seasonal variation in oestrogenic potency and biological effects of effluents from three different wastewater treatment works.

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2.1 Abstract

Effluents from wastewater treatment works (WwTW) have been shown to exhibit both temporal and spatial variation in oestrogenicity, however few studies have attempted to quantify how this variation affects biological responses in fish.

In this study, a novel oestrogen-responsive green fluorescent protein (ERE-GFP) transgenic zebrafish model was applied to quantify for health effects and for oestrogenic biological activity in three different WwTW effluents at 4 time points over a 12-month period. Zebrafish embryos were exposed from fertilisation to 96 hours post fertilisation to either a WwTW effluent at 100% or 50% dilution or a reference oestrogen positive control (17α-ethinyl-estradiol [EE_2]). Measured endpoints included survival/hatching rate, GFP induction (measured in target tissues such as the liver via fluorescence microscopy, and via Western blotting or mRNA induction in whole embryos) and *vtg* mRNA induction.

In wastewater effluent exposures, biological effects such as increased mortality (observed at one of the WwTWs only), decreased hatching rates and up-regulation of *vtg* mRNA were observed and these corresponded well with variations in the measured levels of steroidal oestrogens found in the different effluents over the different study periods. Lowest-observed-effect concentrations (LOECs) for *vtg* induction for the three different WwTWs ranged from 31 ng/L^{-1} EEQs, to 39 ng/L^{-1}.In addition, tissue-specific patterns of GFP expression were identified amongst the three WwTW effluents and were reflective of the known target tissues for the steroidal oestrogens present and for some unidentified chemicals likely present (i.e. nonylphenol or BPA). Furthermore, when compared with GFP induction these markers were similarly responsive to the measurement of *vtg* mRNA, a well-established biomarker for oestrogen exposure.

Overall, this study demonstrates how an integrated approach, supplementing analytical chemistry with a biosensor transgenic zebrafish and effect-based analysis, can be effective for providing information on the potential for differing health effects even for a single WwTW effluent over time/season, using an adverse outcome pathway framework.

2.2 Introduction

Evidence from both field and laboratory studies have shown a clear link between exposures to Wastewater Treatment Work (WwTW) effluents and adverse health effects in aquatic wildlife. In fish, these effects include altered reproductive development (Thorpe *et al.,* 2009; Tetreault *et al,* 2012), genotoxic damage (Liney *et al.,* 2006; Filby *et al.,* 2007; Rocco *et al.,* 2012), alterations in immune function (Hoeger *et al.,* 2004; Liney *et al.,* 2006), cytotoxic and oxidative damage (Gagne *et al.,* 2006) nephrotoxicity (Liney *et al.,* 2006) and decreased body condition/growth (Schäfers *et al.,* 2007; Shved *et al.,* 2007, 2008; Xu *et al.,* 2008). Discharges of WwTW effluents and the chemicals they contain may, in some circumstances, therefore impact adversely on individual fish health with the potential for population level effects.

Globally, it is estimated that over 450 km^3 of domestic, industrial and agricultural wastewater is released into our aquatic environments each year, which in turn requires 6000 km^3 of freshwater for treatment – an amount equivalent to two thirds of the world's usable surface freshwater (Shiklominov, 2000). Dilution of these wastewaters varies both regionally and seasonally, and, in some instances, effluents can be released undiluted (and even untreated) into natural freshwaters (Corcoran, 2010). As the need for clean water is ever increasing, with a six-fold rise in demand over the past century (Mace, 2006), this will put further pressures on the use of freshwater for the dilution of effluent discharges. Potential threats of WwTW effluents to aquatic wildlife becomes of greater global significance when the uncertainty of the long-term stability of our freshwater reserves is recognised due to other factors such as habitat loss and climate change.

Effluents from WwTWs contain highly complex mixtures of chemicals, comprising mixtures of natural and synthetic organic substances, metals and trace elements, making it inherently difficult to establish specific chemical cause and effect relationships for the effects seen in exposed aquatic wildlife. Disruptions in some reproductive processes in fish exposed to these effluents, including, altered sex hormone levels (Sepulveda *et al.,* 2004; Hoger *et al.,* 2006; Jeffries *et al.,* 2008), the induction of intersexuality (Vajda *et al.,* 2008; Williams *et al.,* 2009; Bahamonde *et al.,* 2015a/b) and decreased gamete production and reduced fertility in fish (Hewitt *et al.,* 2008; Tetreault *et al.,* 2012) have been shown to occur as a consequence of the presence of endocrine disrupting chemicals (EDCs). The concept that exogenous substances can interfere

with endogenous hormones in the body responsible for the regulation of essential physiological processes was reported as far back as the 1930/40's (Walker and Janney, 1930; Cook *et al.,* 1934; Sluczewski and Roth, 1948), however, the more recent, and accumulating evidence for exposure effects in both wildlife and humans has placed some EDCs as potentially a major health issue. Some EDCs are now classed as priority substances with regards to water pollution (Defra, 2015).

Some of the most potent EDCs found in WwTW effluents are steroidal oestrogens (both natural and synthetic), with many studies confirming their ability to disrupt sexual development and reproduction in freshwater fish, including at environmentally relevant exposures (Nash *et al.,* 2004; Brian *et al.,* 2006; Kidd *et al.,* 2007; Schwindt *et al.,* 2014). It has been suggested also that exposure to oestrogenic chemicals in humans can induce adverse reproductive health effects, including reduced sperm count/quality, disruptions in the development of the reproductive tract and oestrogen dependent cancers (Talsness, 2009; Li, *et al.,* 2011) as well as wider health effects on the immune system, obesity and cardiovascular disease (Lang *et al.,* 2008; Sweeny, 2009; Hatch *et al.,* 2010).

The oestrogenic activity of WwTW effluents is highly variable and can be influenced by several factors, including the composition of the wastewater entering the works and the type of treatment process employed. For example, certain types of industrial wastewater (such as that generated from textile manufacture) can contain alkylphenolic chemicals and their degradation products (i.e. nonylphenol), at concentrations that induce a strong oestrogenic response (in exceptional circumstances in tens to hundreds of u/L, although more normally at a few ug/L; Sheahan *et al.,* 2002). The oestrogenic activity of WwTW effluents can also be affected by a variety of seasonal changes. For example, at times of high level rainfall, wastewater will be more diluted, whilst in temperate climates during warmer periods microorganisms can be more efficient in biodegrading chemicals within the WwTWs, resulting in low level discharges for some of the EDCs of concern (Cruikshank and Gilles, 2007; Phillips *et al.,* 2012).

Various assay systems have been adopted for the screening and monitoring of environmental oestrogens in WwTWs, including both *in vitro* and *in vivo* tests. *In vitro* tests are generally more easily and arguably efficiently applied. They have included the yeast oestrogen screen (YES) assay and E-Screen assay which involve a single

oestrogen receptor (ER) subtype of the two ERs that occur in humans (ERα and ERβ; three forms occur in fish - ERα, ERβ1 and ERβ2) (Legler *et al*., 1999; De Boever *et al.,* 2001). These assays provide information on levels of oestrogen activity at the level of a single receptor interaction site but are clearly limited and extrapolation to possible health effects is extremely difficult. *In vivo* test systems for WwTw effluents have focused mainly on reproduction and they extend from the use of biomarkers, such as vitellogenin, to egg production in the more complex test systems. They do not however necessarily capture the wider range of possible health outcomes associated with exposure to oestrogens.

The need for an alternative, effect-based approach therefore exists and is increasingly important, particularly in terms of regulatory assessment of wastewater effluents. The application of transgenic fish models offers huge potential for more integrative health effects assessments in both mixture and temporal effects analyses (Brion *et al.,* 2012; Gorelick *et al.,* 2014; Li *et al.,* 2017) and for tailoring future approaches to environmental risk assessment strategies, specifically in respect of adverse outcome pathway (AOP) frameworks. In particular, the linkage between the binding and activation of the oestrogen receptor (ER), known as a molecular initiating event (MIE), and subsequent 'key events' (KEs) in the toxicity pathway (at the cellular and organ level), leading to an adverse outcome (AO) at the organism and population level (Ankley *et al.,* 2010) can be evidenced or inferred.

Several transgenic biosensor fish lines have been developed, that offer an effective, simple, rapid, cost-effective and sensitive method for providing novel insights into the potential health impacts of oestrogenic compounds (Bogers *et al.,* 2006; Brion *et al.,* 2012; Lee *et al.,* 2012; Gorelick *et al.,* 2014; Green *et al.,* 2016, 2018).

In previous work, an oestrogen responsive element (ERE) – green fluorescent protein (GFP), transgenic zebrafish model has been developed that shows high sensitivity for detecting oestrogenic responses (for all three ER subtypes), including for environmentally relevant exposure concentrations. Using this model, a broad range of tissues targets for oestrogens has been identified including the heart, forebrain, otic vesicle, muscle, lateral line and nerve ganglions and the responses have been shown to vary for different environmental oestrogens (Lee *et al.,* 2012; Green *et al.,* 2016).

In this study, the biological effect of exposure to three different WwTW effluents, with differing levels of oestrogenic activity, was assessed using an ERE-TG GFP zebrafish, to investigate for responding target tissues and assess how responses varied both seasonally and between the different WwTW effluents. In addition, fluorescence responses in the transgenic zebrafish model were compared, in terms of sensitivity, for the detection of oestrogens (in this instance in a complex environmental mixture), with vitellogenin (VTG) induction, a well-established biomarker for oestrogen exposure in fish.

2.3 Materials and methods

2.3.1 Test Animals and Husbandry. ERE-GFP transgenic zebrafish (developed at the University of Exeter; 3×ERE:Gal4ff and UAS:GFP; Lee *et al.,* 2012) employed in this study (**SI 1**) were raised at 28.5 +/- 1 °C under a 12-h light, 12-h dark cycle photoperiod regime and fed to satiation three times daily with ZM Fry food 000/100 (Zebrafish Management Ltd., Wincester, U.K.) and Liquifry No. 1 (Interpet, Dorking, U.K.).

2.3.2 Water supply. The supply of water to the laboratory aquaria was subjected to reverse-osmosis with subsequent the addition of salts (as described in OECD Guideline 203). Water temperature and pH levels were monitored daily and ranged between 26.5 and 27.6 °C in all experimental exposures, with pH levels between 6.8 and 7.7. Dissolved oxygen concentrations were maintained at >80% of the air saturation value throughout all exposures. Conductivity of the test water ranged between 200 and 255 µS/cm.

2.3.3 Study WwTWs effluents. All WwTWs are routinely ascribed a total population equivalent or unit per capita loading (PE). PE is a value used by water companies as a means of quantifying the organic strength of wastewater discharges and reflects the net waste of the surrounding human population and industrial facilities (in European Union countries this value is based on a biological oxygen demand (BOD) of the water of 60 g per person/per day). In terms of steroid oestrogen prediction, it is therefore important to know the PE associated with the studied WwTW. For this study, both total and industrial PEs for each of the WwTWs - Countess-Wear (Exeter), Tiverton and Plymouth (Plymouth Central) were obtained from the water utilities responsible for their operation, i.e. South West Water. All WwTWs used in the study are typical of those found in the UK, in that they receive waste primarily from domestic sources, together with some industrial/trade waste. Full details of total population equivalent, types of treatment processes and the types of industrial/trade discharges at each site are listed in **Table 1.** Dissolved oxygen concentrations for all consignments of effluent were above 80%, with pH values for the effluent consignments ranging between 6.5 and 7.2 for Exeter, 6.9 and 7.3 for Plymouth and 7.4 and 7.7 for Tiverton WwTW. Conductivity ranged from 590-980 µS/cm for Exeter, 4122-7173 µS/cm for Plymouth and 460-590 µS/cm for Tiverton WwTW. A range of physiochemical parameters were measured, including temperature, rainfall, dissolved/suspended solids, nitrogen and phosphorus levels, and

biochemical and chemical oxygen demand (BOD/COD) concentrations and are listed in **Table 1**. Differences in each of these parameters over all sampling points were assessed and compared with the levels of oestrogens measured at the same period.

Samples collected for analytical chemistry and fish exposures comprised of 24-hour composite samples (10 L per sampling occasion) collected three times a week from the study WwTWs. Samples were collected under variable weather flow conditions using an automatic sampler, in which the sampling frequency was set to every 20 mins (i.e. 72 individual grab samples in total/24h). All samples were subsequently transferred to 2.5 L sterile glass Winchester bottles, that has been previously acid washed and rinsed with acetone (Analytical reagent grade) and dichloromethane (HPLC grade), to remove any traces of contaminants, and immediately transported back to the university in ice boxes. Upon arrival at the lab, samples needed for the fish exposure work were slowly acclimated to the desired test temperature of 28 °C, stirred and aerated 1hr prior to exposure, and remaining samples were stored at 4 °C to minimise the risk of oestrogen degradation until required for renewal of the exposure medium.

2.3.4 Analytical measurement of concentrations of oestrogenic chemicals; Estrone (E_1), 17β-estradiol (E_2), 17α-ethinyl-estradiol (EE_2). Analytical chemistry was conducted for all WWTW sample collections. For this, 3 L of effluent samples were spiked immediately upon collection of the WwTW effluent with a 5% methanol/ 1% acetic acid mix and stored at 4 °C prior to Solid Phase Extraction (SPE). 1 L samples of these effluents were then were spiked with an internal standard (0.05% of an oestrogenic mixture containing E_1d4, E_2d4 and C13NP at 2 µg/ml, i.e. 100 ng total and EE_2D4 at 0.5 µg/ml, i.e. 10 ng) and filtered to remove any large particulates, prior to extraction onto preconditioned (with HPLC-grade methanol and water) 1 g Oasis HLB cartridges. The cartridges were then eluted with 20 mL 95:5 methanol:water, rinsed with HPLC water to remove any salts and then evaporated to dryness under vacuum for subsequent measurement of E_1, E_2, EE_2, concentrations (via UHPLC-MS/MS). All positive controls (EE_2) and dilution water controls were treated and extracted under the same conditions. Ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analyses was carried out using a Waters Acquity UHPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). The limits of detection were 0.35, 0.25, and <0.5

ng/L for E_2, E_1, and EE_2, respectively. Details of the LC-MS Method can be found in Supplementary Information (**SI 2**).

2.3.5 Estimation of the relative oestrogenic potencies of the WwTW effluents tested (based on the measurements of E_1, E_2, and EE_2). The relative potencies of $1E_2$:$0.75E_1$:$40EE_2$ was used to calculate the equivalent concentrations of E_2 (E_2 EQs) of the WwTW effluents for use in comparing the total steroidal oestrogenic content with responses for GFP and VTG induction (levels of their respective mRNA) in the exposed ERE-GFP zebrafish. These relative oestrogenic potencies were chosen based on information in the literature for the induction of VTG in zebrafish (*Danio rerio*) (Van den Belt *et al.*, 2004; Jobling *et al.,* 2006). This toxic equivalency approach is a recommended approach for assessing the risk of oestrogenic chemical mixtures, such as wastewater effluent, and is based on the assumption that the combined effects of endogenous oestrogens are additive in their effects (Thorpe *et al.,* 2001, 2006).

2.3.6 Experimental Exposures. ERE-GFP zebra fish embryos (*n*= 50 per treatment x 3 replicates in 400 ml sterile glass dishes) were exposed from fertilisation to 96 hours post-fertilisation (96 hpf) to one of the following treatments: a dilution water control (DWC), a reference oestrogen positive control (EE_2, at nominal concentration of 10 ng/l) or a WwTW effluent at either 100% or 50% dilution with embryo culture water (prepared in accord to ISO guidelines). Stock solutions of EE_2 (≥ 98% purity, purchased from Sigma, Poole, UK) were prepared in acetone (10 mg/l) and stored in a dark cold-room (4 °C) to minimise the risk of degradation. Solvent free working solutions were prepared (in embryo culture water) the day before use, by pipetting the required amount of stock solution into a glass duran bottle and evaporating the solvent away under a stream of nitrogen. In the embryos cultures, 50% of the treatment medium was replaced every 24 hours and all treatments were gently aerated using a glass pipet connected to tubing fed through each dish lid to ensure that the dissolved oxygen concentration remained greater than 80% of the air saturation value.

2.3.7 Fish sampling and analysis of phenotypic endpoints. All animal use protocols were carried out ethically in accordance with U.K. Home Office guidelines (Animals (Scientific Procedures) Act 1986). Fish were sacrificed by terminal anesthesia with benzocaine (0.5 g/L; ethyl-*p*-aminobenzoate; Sigma, Poole, U.K.). Triplicate sets of embryos were sampled at 96 hpf and examined for GFP expression using Western blot analysis (*n*= 10 fish per treatment) and image analysis (*n*= 15 fish per treatment) and

for VTG/GFP mRNA expression (*n*= 15 fish per treatment). Other measured endpoints included survival and hatching rate (success).

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

2.3.9 Quantification of GFP (EGFP) expression using image analysis. GFP expression for the individual tissues was quantified using ImageJ™ software (http://rsb.info.nih.gov/ij/). For each image, tissues of interest were masked (outlined) manually to provide a specific quantifiable region of interest (ROI). The mean pixel intensity value from the ROI was then used as a means of quantifying the fluorescence response, and this was kept consistent between individuals. Background was subtracted using the ImageJ rolling ball algorithm which removes any spatial variations of the background intensities as described in Sternberg (1983). For quantification in the somites, three random measurements were taken employing a plug-in Python macro.

2.3.10 Quantification of GFP (EGFP) expression using Western blot analysis. All fish were sampled at 96 hpf to quantify who body GFP expression using Western blot analysis, according to the protocol described previously (Lee *et al.,* 2012) with the strength of the GFP signal evaluated with Image J software (http://rsbweb.nih.gov/ij/) standardized to the intensity of alpha tubulin and shown as a fold increase in GFP levels in exposed embryos over unexposed embryos.

2.3.11 Quantification of the levels of GFP mRNA and VTG mRNA in whole embryo-larvae using RT-qPCR.

2.3.11.1 RNA extraction and cDNA synthesis. Samples for measurement of whole body VTG and GFP mRNA were immediately snap-frozen in liquid nitrogen upon collection and stored at −80 °C until use. Total RNA was extracted from all samples using Trizol Reagent (Sigma), according to the manufacturer's instructions. The amount of RNA was quantified using a NanoDrop 1000 spectrophotometer and RNA purity determined through the measurement of the A260/A280 and A260/230 ratios, respectively. Reverse transcription was subsequently carried out by incubating 1 µg RQ1 DNase treated (Promega) total RNA with 5 mM random hexamers (Eurofins MWG Operon) 10 mM dNTPs and 1 µl MMLV-Reverse transcriptase (Promega) in the appropriate buffer for 60 min at 37 °C and 10 min at 42 °C. Polymerase chain reaction (PCR) was performed in an iCycler hermocycler coupled to a MyiQ detector (Bio-Rad. Hercules, CA, USA).

*2.3.11.2 Real-time quantitative PCR***.** RT-qPCR was carried out on cDNA samples for each treatment group and sampling point for selected target genes with the iCycler iQ Real-time Detection System (Bio-Rad Laboratories Inc., CA, USA) using the following primers:

Vtg1 (fw) 5'-GCCAAGAAAGAACCCAAACTG-3', Vtg1 (rev) 5'-GGAGATGAGAGCCACTGAAG-3';
EGFP (fw) 5'-CGACGGCAACTACAAGAC-3', EGFP (rev) 5'-TAGTTGTACTCCAGCTTGTGC -3'.

Primer sequences were either designed with Beacon Designer software (Premier Biosoft International, Palo Alto, USA) or using previously published sequences, and obtained from MWG-Biotech (Ebersburg, Germany). In brief, primer pairs were optimized for annealing temperature (Ta), specificity confirmed by melt curve analysis, and the detection range, linearity and amplification efficiency (*E*) established using serial dilutions of zebrafish cDNA. RT-qPCR was carried out using Absolute QPCR SYBR Green Fluorescein mix (ABgene), with an initial activation step of 95 °C for 15 min followed by 40 cycles of denaturation (95 °C, 10 s) and annealing (appropriate Ta, 45 s) and final melt curve analysis. Expression levels of ribosomal protein L8 *(rpl8)* were used as a 'housekeeping' gene to normalize the expression of other genes using a

development of efficiency correlated relative quantification as described previously (Filby and Tyler, 2005).

2.3.12 Statistical analysis

In all cases, data were checked for normality using the D'Agostino-Pearson test and for homogeneity of variance using Bartlett's or Levene's test prior to statistical analysis. Data meeting these tests were analysed using parametric tests, e.g. analysis of variance (ANOVA) procedures, whilst data that did not conform to normality were subject to nonparametric tests, e.g. Kruskal–Wallis. Post hoc analysis was performed against controls using the Dunnett's test and with the Tukey test for between-treatment comparisons. Data were transformed, where necessary, using log10. Any significant differences from expected sex ratios or stages of gonadal development were tested using the chi-square test. All statistical analyses were run in GraphPad Prism 7 (GraphPad Software, Inc., San Diego, USA). Statistical significance was accepted as $p < 0.05$ for all comparisons and values are quoted as mean values and ranges as standard error of the mean.

2.4 Results

2.4.1 Measured concentrations of major steroidal oestrogens in the test effluents.

Analytical measurements for E_1, E_2 and EE_2 (the three principal oestrogens occurring in WwTW effluent) were used to characterise the steroidal oestrogenic content of the three different effluents. Measured concentrations of E_1 and E_2 found in all three WwTW effluents varied across the different collection time-points (**Table 1**). At Exeter WwTW, concentrations of E_1 ranged between 14.0 and 18.41 ng/L during March 2013 (mean 16.7 ± 1.1 ng/L) and were considerably lower during Oct 2013 (between 0.4 and 1.3 ng/L) and Jan 2014 (mean 0.8 ± 0.2 ng/L). E_2 concentrations ranged between 0.4 and 2.4 ng/L (mean 0.7 ± 0.3 ng/L). The level of total oestrogenic activity for this WwTW effluent ranged between 18.8 ng/L^{-1} EEQs to 33.1 ng/L^{-1} EEQs.

Plymouth WwTW was the most oestrogenic of the effluents tested, where concentrations of E_1 ranged between 23.4 and 56.9 ng/L during Jan 2014 (mean 36.7 ± 4.1 ng/L) and were at their lowest (between 0.2 and 1.3 ng/L) during July 2013 (mean 0.6 ± 0.2 ng/L). E_2 concentrations varied only between 30 and 32 ng/L (mean 32 ± 0.4 ng/L) during Jan 2014, with the lowest levels at between 0.2 and 1.2 ng/L (mean 0.5 ± 0.4 ng/L) during Oct 2013. Levels of total oestrogenic activity measured at this site ranged between 19.1 ng/L^{-1} EEQs and 85.6 ng/L^{-1} EEQs. Measured concentrations of E_1 from the different consignments of effluent collected from Tiverton WwTW ranged between 7.8 and 14.5 ng/L during April 2014 (mean 11.4 ± 1.2 ng/L) and were at their lowest, at between 0.2 and 4.7 ng/L, during July 2014, (mean 1.9 ± 0.8 ng/L). E_2 concentrations varied between 1.2 and 4.6 ng/L (mean 2.4 ± 0.9 ng/L) during April 2014, and were lowest, at between 0.3 and 0.5 ng/L (mean 0.4 ± 0.03 ng/L) during Jan 2014. Levels of total oestrogenic activity measured at Tiverton WwTWs ranged between 22 ng/L^{-1} EEQs and 31 ng/L^{-1} EEQs. The synthetic oestrogen, EE_2 was detected intermittently at all WwTWs (up to 1.5 ng/L; limit of detection = 0.5 ng/L). EE_2, E_2 and E_1 were below the minimum detection level (i.e. 0.5; 0.4; 0.2 ng/L, respectively) in all control water samples. Chemical analysis of the positive control EE_2, showed measured concentrations ranging from between 70 - 130% of the nominal test concentration (mean value, 7.8 ng/L).

Site	Exeter Countess Wear WwTW				Plymouth Central WwTW				Tiverton Allers WwTW			
Total population equivalent	141698				117574				23648			
Secondary treatment	Activated sludge				BAFF				Biological filter			
Tertiary treatment	Nitrifying BAFF UV Disinfection				UV Disinfection				None			
Physiochemical parameter	**Mar-13**	**Jul-13**	**Oct-13**	**Jan-14**	**Mar-13**	**Jul-13**	**Oct-13**	**Jan-14**	**Oct-13**	**Jan-14**	**Apr-14**	**Jul-14**
pH	6.5 ± 0.07	7.0 ± 0.05	7.0 ± 0.06	7.2 ± 0.10	7.3 ± 0.05	6.9 ± 0.06	7.2 ± 0.05	7.3 ± 0.09	7.4 ± 0.05	7.7 ± 0.09	7.5 ± 0.05	7.6 ± 0.03
rainfall (mm)	0.0	0.0	15.4 ± 12.8	13.1 ± 1.2	0.0	5.3 ± 6.2	32.3 ± 16.5	16.9 ± 4.7	32 ± 13.5	16.5 ± 4.7	0.0	20.3 ± 12.6
Suspended solids mg/l	24 ± 0.76	15 ± 1.45	15 ± 1.61	7 ± 0.94	21 ± 1.21	9 ± 0.93	14 ± 1.57	23 ± 1.24	9 ± 0.83	21 ± 1.23	16 ± 1.37	10 ± 1.69
BOD mg/l	6 ± 0.87	11 ± 0.52	7 ± 0.34	3 ± 0.76	11 ± 0.58	7 ± 0.23	12 ± 0.18	6 ± 0.44	13 ± 0.92	9 ± 0.67	7 ± 0.26	13 ± 0.58
COD mg/l	63 ± 0.52	74 ± 0.77	56 ± 0.62	23 ± 0.91	67 ± 0.25	80 ± 0.69	70 ± 0.87	53 ± 0.64	54 ± 0.88	48 ± 0.64	55 ± 0.72	45 ± 0.82
NH3 mg/l	2 ± 0.18	8 ± 0.06	6 ± 0.10	8 ± 0.07	n/a	n/a	n/a	n/a	1.6 ± 0.16	0.4 ± 0.07	0.2 ± 0.05	1.2 ± 0.14
Cl- mg/l	n/a	n/a	n/a	n/a	1491 ± 41.7	2454 ± 31.1	3189 ± 55.4	1777 ± 59.6	n/a	n/a	n/a	n/a
E1 ng/l	16.7 ±1.11	1.8 ±0.19	1.3 ±0.07	0.7 ±0.07	9.0± 0.97	1.1± 0.19	1.0± 0.35	36.7± 6.04	2.2±0.98	4.3±1.90	11.4±1.22	1.9±0.83
E2 ng/l	0.6 ±0.23	1.0 ±0.35	0.5 ±0.15	0.6 ±0.10	4.0± 0.09	1.3± 0.35	1.5± 0.16	32.0± 0.37	0.4±0.08	0.4±0.04	2.4±0.86	0.7±0.18
EE2 ng/l	0.5 ±0.05	0.4 ±0.05	0.5 ±0.04	0.5 ±0.04	0.7± 0.16	0.4± 0.05	0.5± 0.04	0.7± 0.19	0.5±0.07	0.5±0.04	0.5±0.06	0.5±0.02

BAFF = Biologically Aerated Flooded Filter

Table 1. Data on the WwTWs at Exeter, Plymouth and Tiverton. Details include the treatment processes used at each treatment works (including total hydraulic retention times (HRT), sludge retention times [SRT]) total population equivalent (p.e.) values for each site and types of industrial discharges. Physiochemical data includes concentrations of the major steroidal oestrogens found in the test effluents, taken from each of the WwTW study sites during all experimental periods. Data are reported as mean ± SEM.

2.4.2 Responses of the ERE-GFP zebrafish to the WwTW effluent exposures

2.4.2.1 *Embryo survival and hatching success (0-96hpf).*

There were no significant effects on survival for exposures to EE_2 (10 ng/L) (**Fig 1A-Ci**). For effluent exposures no significant effects were seen at Exeter and Tiverton WwTW (**Fig 1Ai, 2Ci**). Exposure to Plymouth WwTW exposures resulted in a lower survival rate during March 2013 (45/90 = 50%, p =<0.001) and Jan 2014 (59/90 = 66%, p=<0.05; **Fig 1Bi**). As all surviving embryos in control groups across all treatments had hatched by 72hpf, this was subsequently used to determine hatching success in other treatments groups.

Exposures to EE_2 (10 ng/L) resulted in a reduced hatching in all cultures conducted (with reduction between 55 and 72%; **Fig 1A-Cii**). Hatching success in embryos exposed to 100% effluent was significantly reduced at all sampling periods for the Exeter WwTW effluent (**Fig 1Aii**). There were also significant differences in the hatching rate for embryos exposed to the 50% Exeter WwTW effluent (**Fig 1Aii**) during March 2013 (31/90 = 34%, p =<0.001) and Jan 2014 (37/90 = 41%, p =<0.01). Hatching success in the full strength (100%) Plymouth WwTW effluent was reduced at all time points (ranging between 28% and 70%, **Fig 1Bii**) and for exposure to 50% effluent (**Fig 1Bii**) during March 2013 and Jan 2014 (35/90 = 39%, p =<0.001 and 56/90=62%, p =<0.001, respectively). In addition, in the Plymouth WwTW effluent

(both 50% and/or 100%) onset in hatching was advanced, observed at 48hpf, at all sampling periods for this treatment works (data not shown). Hatching success in the Tiverton WwTW effluent (50% and 100% effluent) was reduced at all time points, with the exception of October 2013 (**Fig 1Cii**).

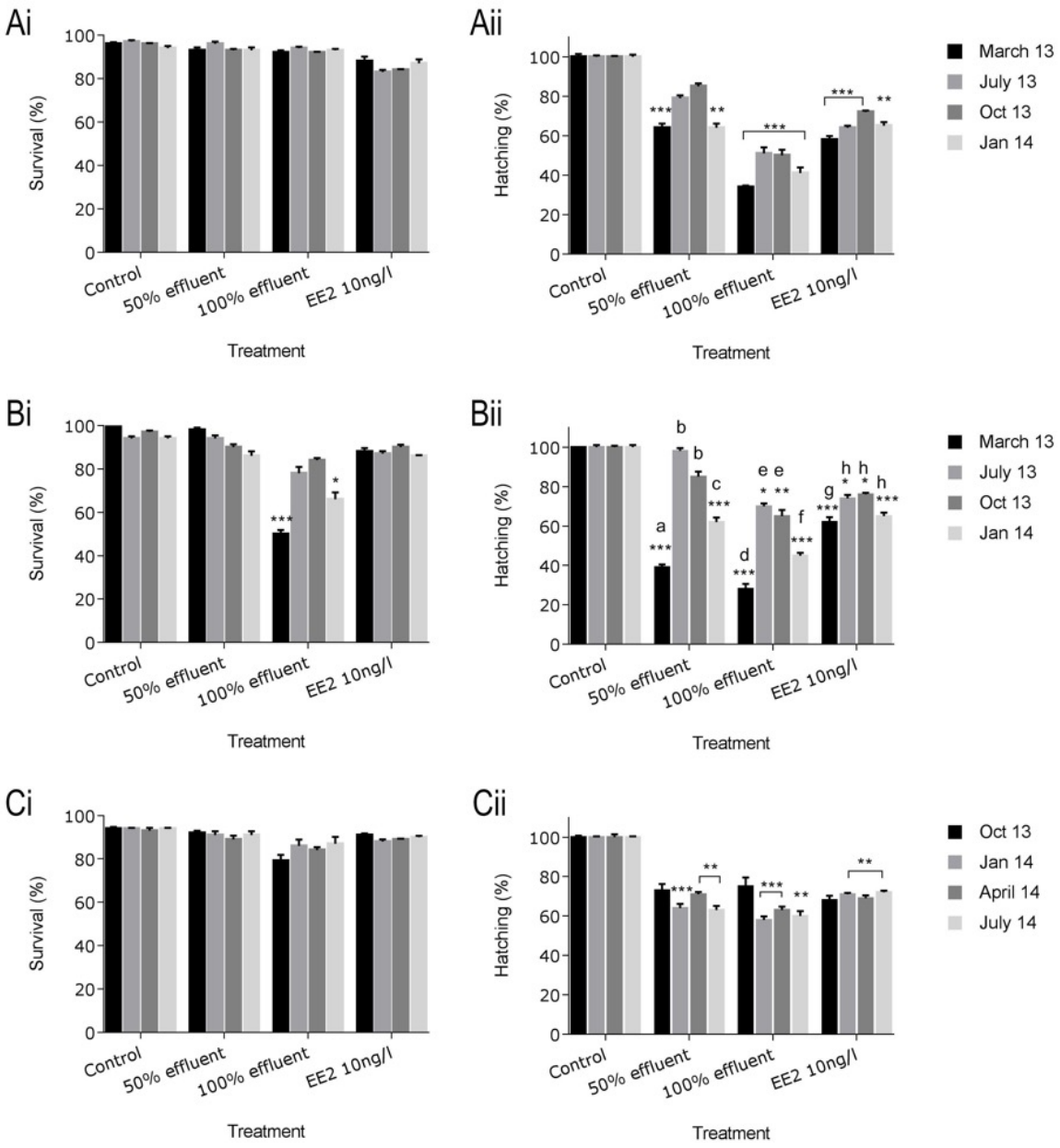

Fig. 1. Hatching rates (at 72hpf) and subsequent survival (at 96hpf) in ERE-GFP zebrafish embryos. Transgenic zebrafish embryos in control (unexposed), EE_2 (10 ng/L, positive control) and effluent (50% or 100%) from (A) Exeter, (B) Plymouth or (C) Tiverton WwTWs. Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. Analysis was conducted three times for any given effluent collection. Data are reported as mean ± SEM. Asterisks denote a significant difference (*p<0.05, **p<0.01, ***p<0.001) compared with control. Different letters denote a significant difference within groups (p<0.05).

2.4.3 GFP expression

2.4.3.1 Tissue response patterns

In some of the control (96hpf) ERE-TG larvae there was a low-level expression of endogenous GFP in both the heart (49% of the fish studied) and in the otic vesicle (56% of the fish studied). In larvae exposed to EE_2 (**Fig. 2Bi-Biii**), GFP expression was observed in the liver, heart, somites, otic vesicle, fins and forebrain. Exposure to full strength (100%) effluent from the three different WwTW resulted in different tissue patterns of GFP expression (**Fig. 2C-Ei-iii**). There was also some variation in the GFP expression within any one study site over the different effluent collections. In embryos exposed to Exeter WwTW 100% effluent, GFP expression was observed in the liver, heart (**Fig. 2Ci-3Cii**) and in the somites (**Fig. 2Ciii**) in March 2013 and in the heart only at all other sampling-points (**Fig. SI 3Dii, 3Eii, 3Fii**). In the Exeter WwTW 50% effluent, GFP expression was observed only in the heart at all sampling points (data not shown). In embryos exposed to 100% Plymouth WwTW effluent, GFP expression was observed in the neuromasts (**Fig. 2Di**) and in the liver, heart and somites in Jan 2014, (**Fig. 2Di-iii**), in the heart and somites in March 2013 (**Fig. SI 3Gii, Giii**) and in the heart only in July and Oct 2013 (**Fig. SI 3Hii; Iii**). In the Plymouth WwTW 50% effluent, GFP expression was observed in the liver and somites in Jan 2014 and in the heart at all other sampling points (data not shown). For Tiverton WwTW exposures, GFP expression was observed in the heart (**Fig. SI 3Lii**) and in the somites (**Fig. SI3Liii**) during Jan 2014 and April 2014 (**Fig. SI 3Mii-iii**) and was also seen in the heart at all other sampling-points (**Fig. SI3 Kii; Nii**). No specific GFP expression was detected in the liver. In the Tiverton WwTW 50% effluent, GFP expression was observed only in the heart at all sampling-points (data not shown).

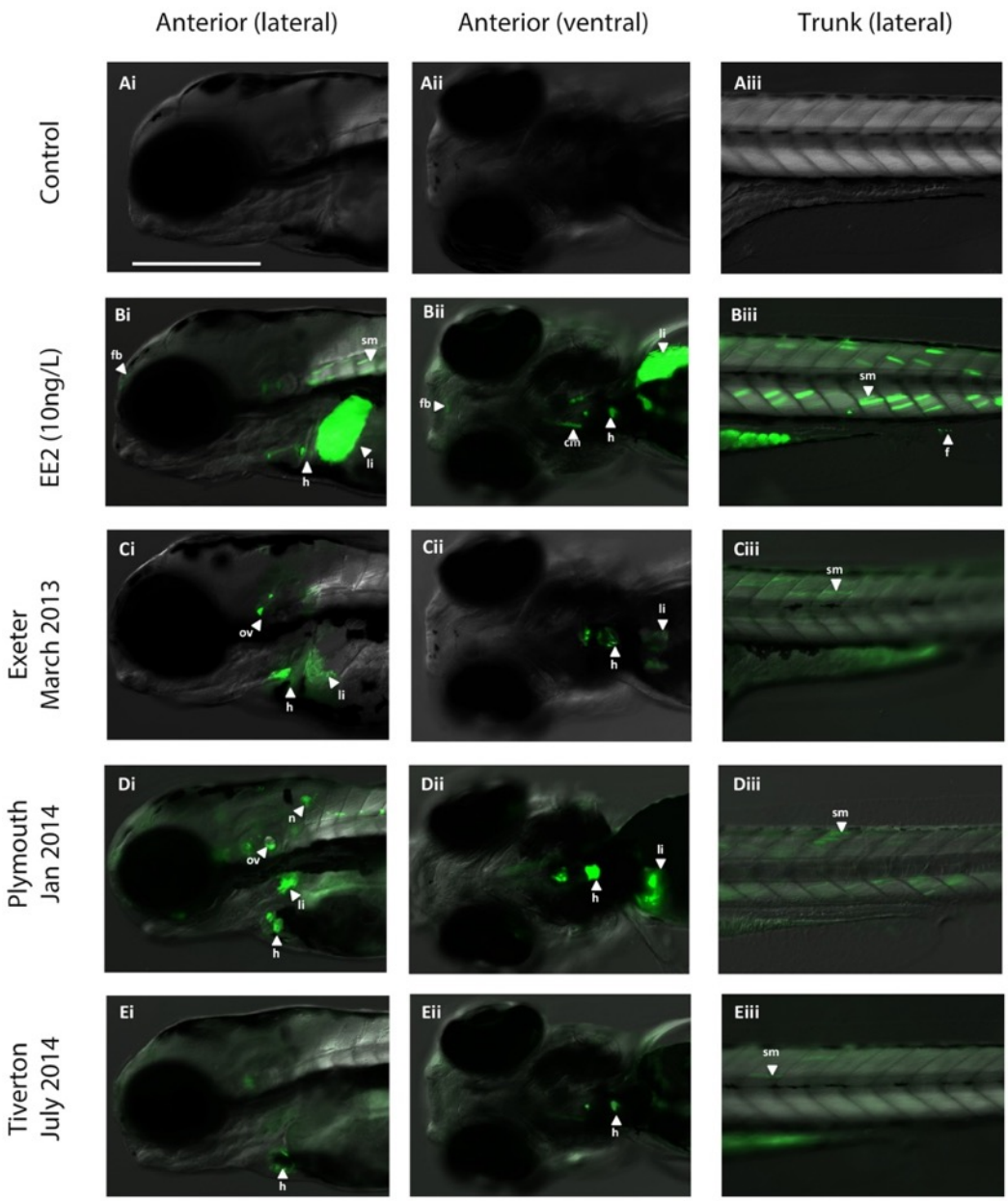

Fig 2. Expression of GFP in different tissues in whole ERE-GFP zebrafish exposed to various wastewater effluents from fertilisation to 96 hours post fertilisation. Transgenic zebrafish larvae in control (unexposed) (**A**), EE_2 (10 ng/L, positive control) (**B**), and wastewater effluent (100%) exposed groups at Exeter WwTw (**C**), Plymouth WwTw (**D**) and Tiverton WwTw (**E**). Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. GFP expression is shown for regions in the head (lateral – I and ventral – ii views) and trunk (lateral view - iii). GFP induction was observed in the heart (h), liver (li), forebrain (fb), otic vesicle (ov), cranial muscles (cm), fin (f), and somite muscles (sm) in the positive control larvae (**B**), but the wastewater effluents induced different tissue patterns of GFP expression (**C-D**). Exeter (C) induced GFP expression in the h, li, ov and sm. In Plymouth-exposed larvae (D), GFP expression was detected in the neuromast (n), li, h, ov and sm. In Tiverton (E) exposed larvae, h and sm expression was observed only. Bar = 200 µm.

2.4.3.2 Quantified tissue responses – image analysis

Quantifying the tissue GFP responses via image analysis, exposure to 10 ng/L EE_2 in all cases produced a significant fluorescence induction in the liver, heart and somites, compared with untreated controls, with an average 82-fold increase in the liver, and ranging between no response and 38-fold increase in the heart and between no response and 50-fold increase in the somites (**Fig. 3A-C**). For the Exeter WwTW, there was a significant GFP induction in the liver in March 2013 in both 50% and 100% effluent (<*p*=0.01 and *p*=<0.0001, respectively; **Fig. 3Ai**). In the heart, there was a significant GFP induction in the 50% and 100% effluent treatments during March 2013 (8.4, *p*=<0.05 and 19.5, *p*=<0.0001) and in the 100% effluent treatment in Oct 2013 (*p*=<0.001; **Fig. 3Aii**). In the March 2013, exposure to 100% Exeter WwTW effluent resulted in a significant GFP induction in the somites (*p*=0.0001; **Fig. 3 Aiii**). For Plymouth WwTW, there was a significant hepatic induction of GFP in Jan 2014 in both 50% and 100% effluent (*p*=<0.0001 and *p*=<0.0001, respectively **Fig. 3Bi**). In the heart, GFP induction was significant at all time-points in both effluent exposure concentrations (50% effluent – *p* values between <0.0001and 0.001; 100% effluent - *p* values <0.0001; **Fig. 3Bii**). For the somites, GFP was significantly elevated in the Plymouth WwTW effluent in Jan 2014 (both 50% and 100% exposures; *p*=<0.0001) and during March 2013 (100% effluent *p*=<0.0001; **Fig. 3Biii**). For the Tiverton WwTW exposures, there was a significant fluorescence induction in the heart at all time points for the 100% effluent exposures (*p* values between <0.01 and 0.0001; **Fig. 3Cii**) and in the 50% effluent exposures at all time-points with the exception of Oct 2013 (*p*=<0.0001; **Fig. 3Cii**). Somite GFP induction was significantly elevated in Jan 2014 (*p*=<0.0001) and April 2014 (*p*=<0.0001) in 100% effluent exposures only (**Fig. 3Ciii**).

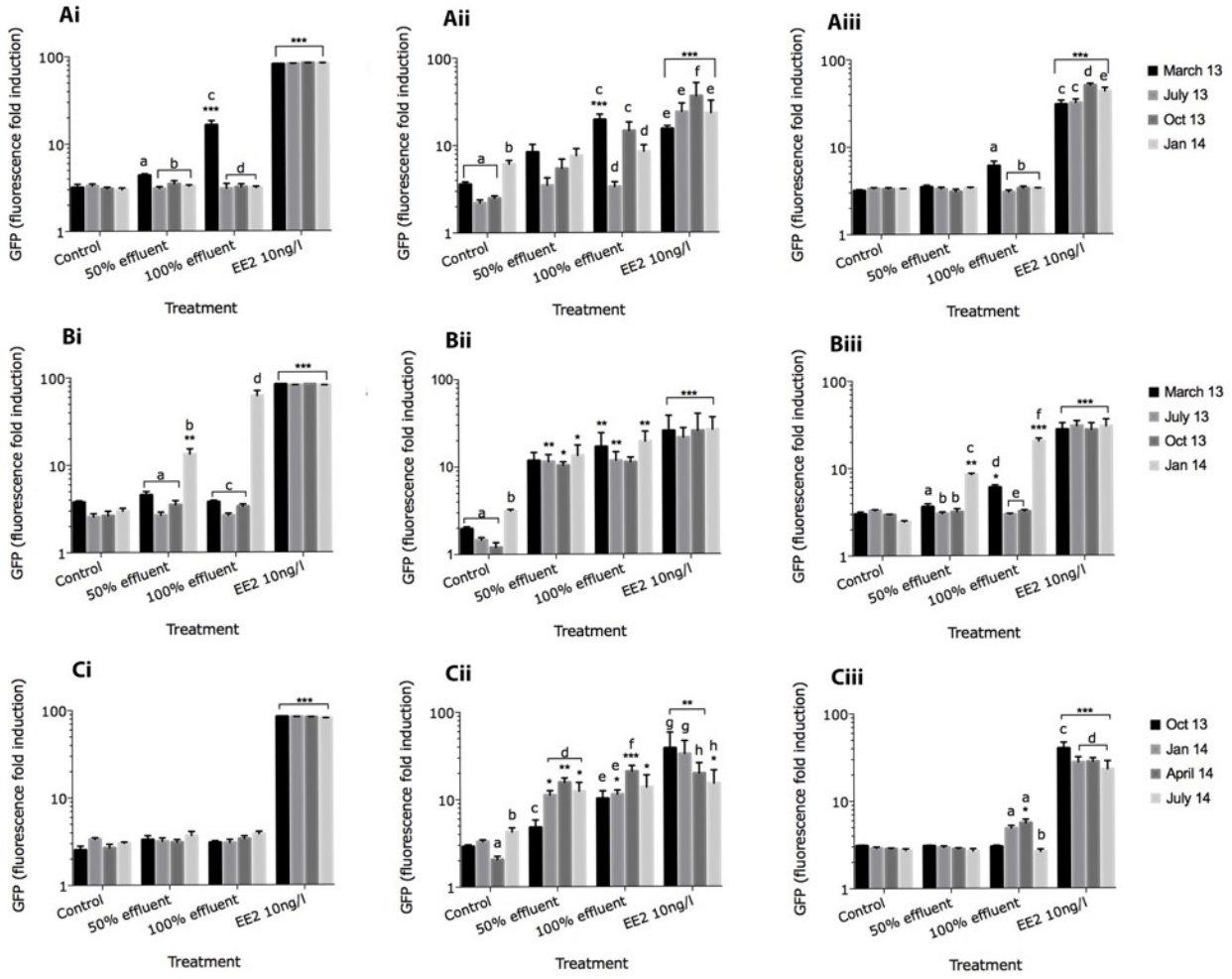

Fig 3. Quantification of target tissue responses in ERE-GFP zebrafish exposed to various wastewater effluents, as determined by fluorescence induction. Data shows GFP intensity fold increase in GFP levels in the liver (i), heart (ii) and somites (iii) in exposed ERE-GFP larvae over unexposed larvae, at Exeter WwTW (A), Plymouth WwTw (B) and Tiverton WwTw (C). Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. Analysis was conducted three times for any given effluent collection. Data are reported as mean ± SEM. Asterisks denote a significant difference (*p<0.05, **p<0.01, *** p<0.001) compared with control.

2.4.3.3 Quantified whole-body responses –GFP Western blotting

Quantifying GFP responses via Western Blot analysis of whole-body embryo extracts confirmed exposure to EE_2 (10 ng/L) resulted in a significant induction for all exposures conducted compared with untreated controls (39-42 fold-increase, p=<0.01, **Fig. 4Ai/Bi/Ci**). The only significant induction of GFP for the Exeter WwTW exposure occurred for the March 2013 100% effluent exposure, where there was an 18-fold GFP induction (**Fig. 4Ai**). For exposures to the Plymouth WwTW GFP induction was significantly elevated in the 100% effluent in March 2013 (**Fig. 4Bi**; 6-fold increase compared with untreated controls) and in the January 50% and 100% effluent

exposures (**Fig. 4Bi**; 8-fold increase, p =<0.05, and 19-fold increase, p=0.05, respectively). For the Tiverton WwTW, a significant GFP induction occurred only in the 100% effluent exposures during April 2014 (10-fold increase compared with untreated controls, p =<0.05, **Fig. 4Ci**).

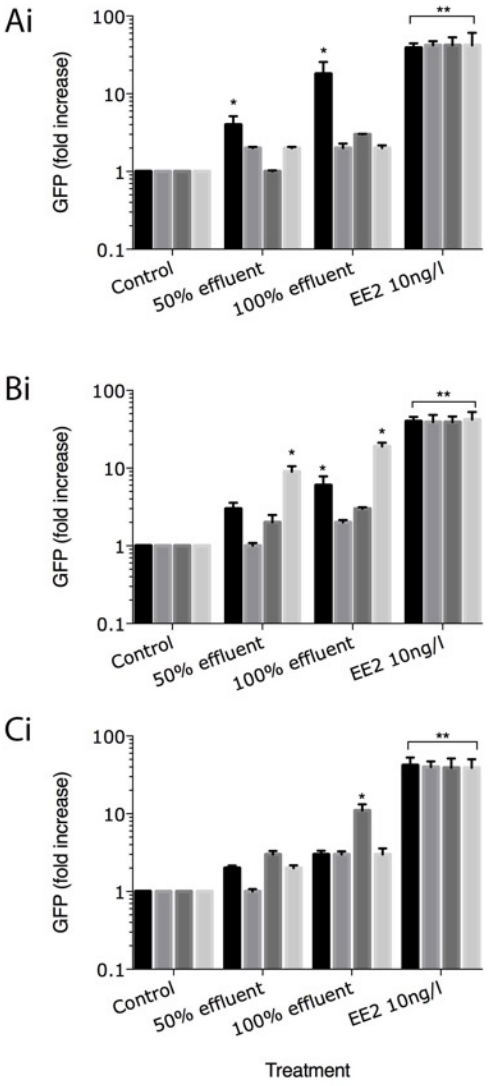

Fig 4. Analysis of GFP expression in whole ERE-GFP zebrafish embryos quantified through Western blotting. Data shows fold increase in GFP expression compared with controls following exposure to EE_2 (10 ng/l) or a WwTW effluent (50% or 100%), at Exeter WwTW (A), Plymouth WwTw (B) and Tiverton WwTw (C). Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. Analysis was conducted three times for any given effluent collection. Data are reported as mean ± SEM. Asterisks denote a significant difference (*p<0.05, **p<0.01) compared with control.

2.4.4 Quantification of vtg mRNA and gfp mRNA expression

Exposure to EE_2 (10 ng/L) induced a 7-11 fold induction of *gfp* and 40-50 fold induction of *vtg*, respectively (p=<0.01, **Fig. 5Aii-iii;Bii-iii;Cii-iii**). For the Exeter WwTW exposures *gfp* mRNA expression appeared to be higher (approximately 3-fold induction) only in embryos exposed to 100% effluent during March 2013, but this was not statistically significant (**Fig. 5Aii**). *vtg* mRNA induction was detected only in embryos following exposure to 100% Exeter WwTw effluent during March 2013 (8-fold induction compared to dilution water controls, p= <0.01, **Fig. 5Aiii**), and was higher also when compared with those measured in July 2013, Oct 2013 and Jan 2014 (p=<0.01, **Fig. 5Aiii**). For *gfp* mRNA expression in the Plymouth WwTW exposures, the only significant induction, compared with dilution water controls, occurred in the Jan 2014 100% effluent sample (a 5-fold induction compared to untreated controls, p=<0.05, **Fig. 5Bii**). *gfp* mRNA levels were also higher in the 100% effluent during Jan 2014 compared with in July and Oct 2013 (p=<0.05, **Fig. 5Bii**). There was a significantly higher level of *vtg* mRNA in embryos following exposure to 100% Plymouth WwTW effluent for both March 2013 and Jan 2014 (3-fold induction, p=<0.05; March 2013 and 22-fold induction, p=<0.001; Jan 2014; **Fig. 5Biii**) compared with dilution water controls. *vtg* mRNA levels were higher in the Jan 2104 100% effluent exposure compared with the 100% effluent exposures at all other time-points (p= <0.001, **Fig. 5Biii**). For Tiverton WwTW, the only indication of an elevation in *gfp* mRNA expression (approximately 2-fold) was for the 100% effluent exposures in Jan and April 2014, but this was not statistically significant (**Fig. 5Cii**). *vtg* mRNA levels were higher in embryos following exposure to 100% effluent during April 2014 only (5-fold induction, p=<0.05, **Fig. 5Ciii**) compared with controls and 100% effluent for the Oct 2013 and Jan 2014 and July 2014 exposures (p=<0.01, **Fig. 5Ciii**).

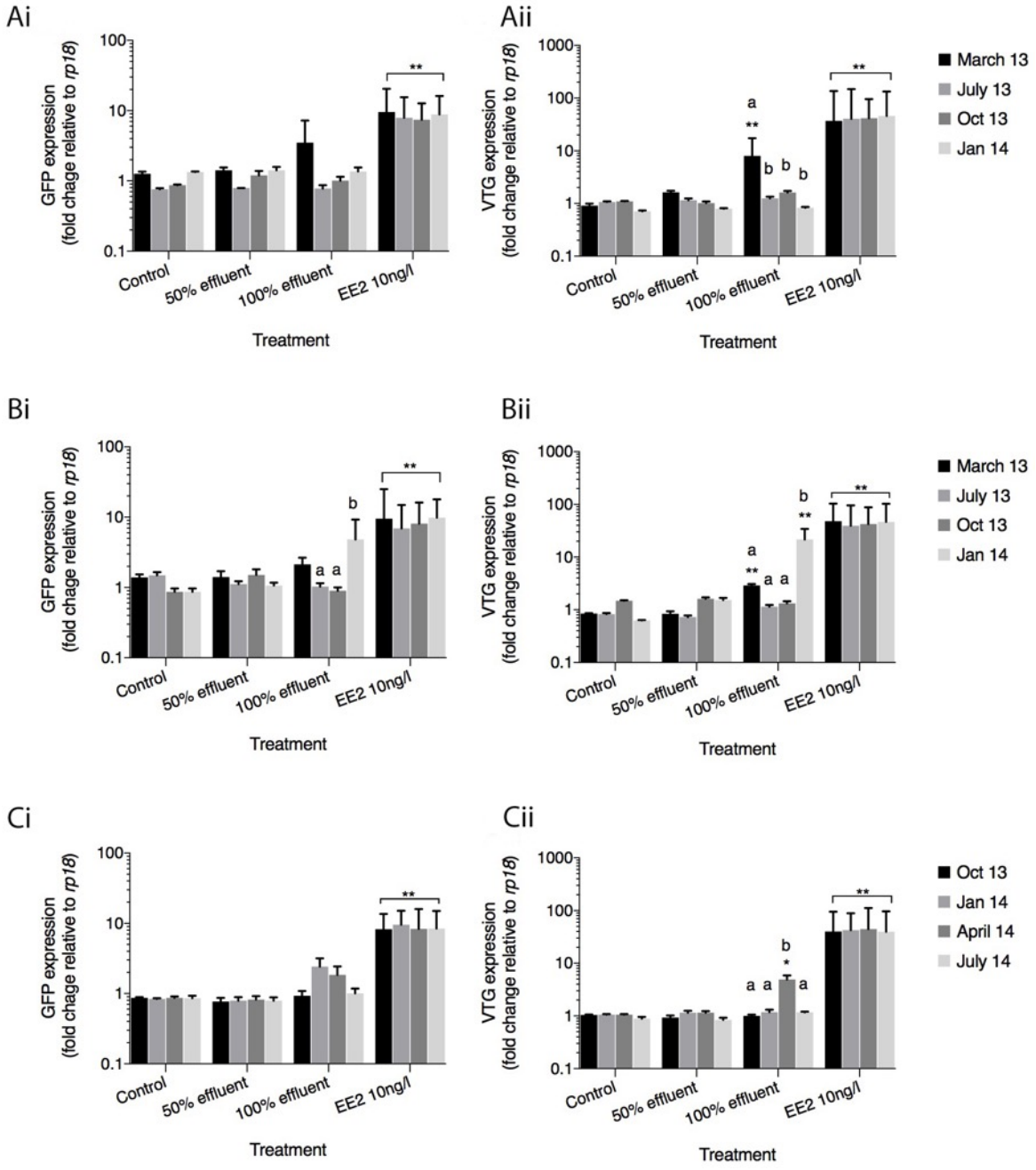

Fig. 5. GFP and VTG mRNA expression in whole ERE-GFP zebrafish embryos. Data shows fold change compared with controls following exposure to EE_2 (10 ng/l) or a WwTW effluent (50% or 100%) at Exeter WwTw (Aii-iii), Plymouth WwTw (Bii-iii) and Tiverton WwTw (Cii-iii). Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. Analysis was conducted three times for any given effluent collection. Data are reported as means ± SEM and expressed as fold-change mRNA levels relative to the reference gene (*rpl8*). Asterisks denote a significant difference (*p<0.05, **p<0.01) compared with treatment control. Different letters denote a significant difference within groups (p<0.05).

In terms of combined threshold responses for the concentrations of the steroidal estrogens E_1 E_2 and EE_2, measured in each of the wastewater effluents, the lowest-

observed-effect concentration (LOEC) for *vtg* induction was 33 ng/L^{-1} EEQs for Exeter WwTW, 39 ng/L^{-1} EEQs for Plymouth WwTW and 31 ng/L^{-1} EEQs for Tiverton WwTW. For GFP expression, the only significant induction, compared with dilution water controls, occurred in Plymouth WwTw exposures, with corresponding LOECs of 27.5 and 32 ng/L^{-1} EEQs.

A comparison of *gfp* mRNA against *vtg* mRNA levels in whole embryo homogenates showed that the magnitude for responses in *vtg* induction was higher compared to *gfp* in both EE_2 (Fig 6A) and wastewater effluent exposures (Fig. 6B). Comparison of the data as a whole revealed a correlation between the levels of *gfp* expression and the levels of *vtg* expression for both effluent and the EE_2 exposures (r^2=0.2, p=<0.05 and 0.5 p=<0.01 respectively; **Fig 6**).

Full details of the analysis of E_2 EQs against the induction of *gfp* and *vtg*, are provided in the Supplementary Information (SI 3).

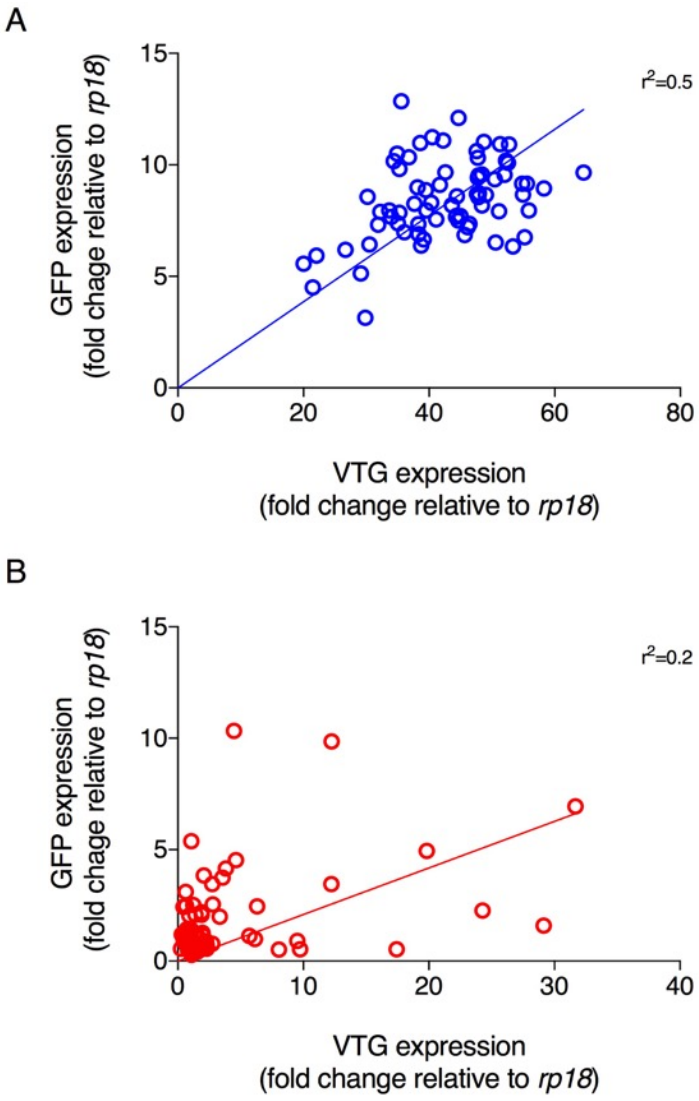

Fig 6. Correlation between GFP and VTG mRNA levels in whole ERE-GFP zebrafish embryos. Data shows (A) EE_2 (10 ng/l, positive control) treatment groups and (B) effluent (50% & 100% combined) treatments groups for Exeter, Plymouth and Tiverton WwTWs. Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. Analysis was conducted three times for any given effluent collection.

2.4.4.1 Temporal responses:

An analysis of time-related GFP induction (% embryos expressing GFP in the heart) showed similar results across the three treatment works. GFP expression was first observed (in both 50% and 100% effluent treatments) in embryos at 48hpf and subsequently at the following levels at 96hpf; Exeter WwTW - 58% and 87%, Plymouth WwTW - 60% and 100%, and Tiverton WwTW - 60% and 97%, in the 50% and 100% effluents, respectively (**Fig 7A, B, C**).

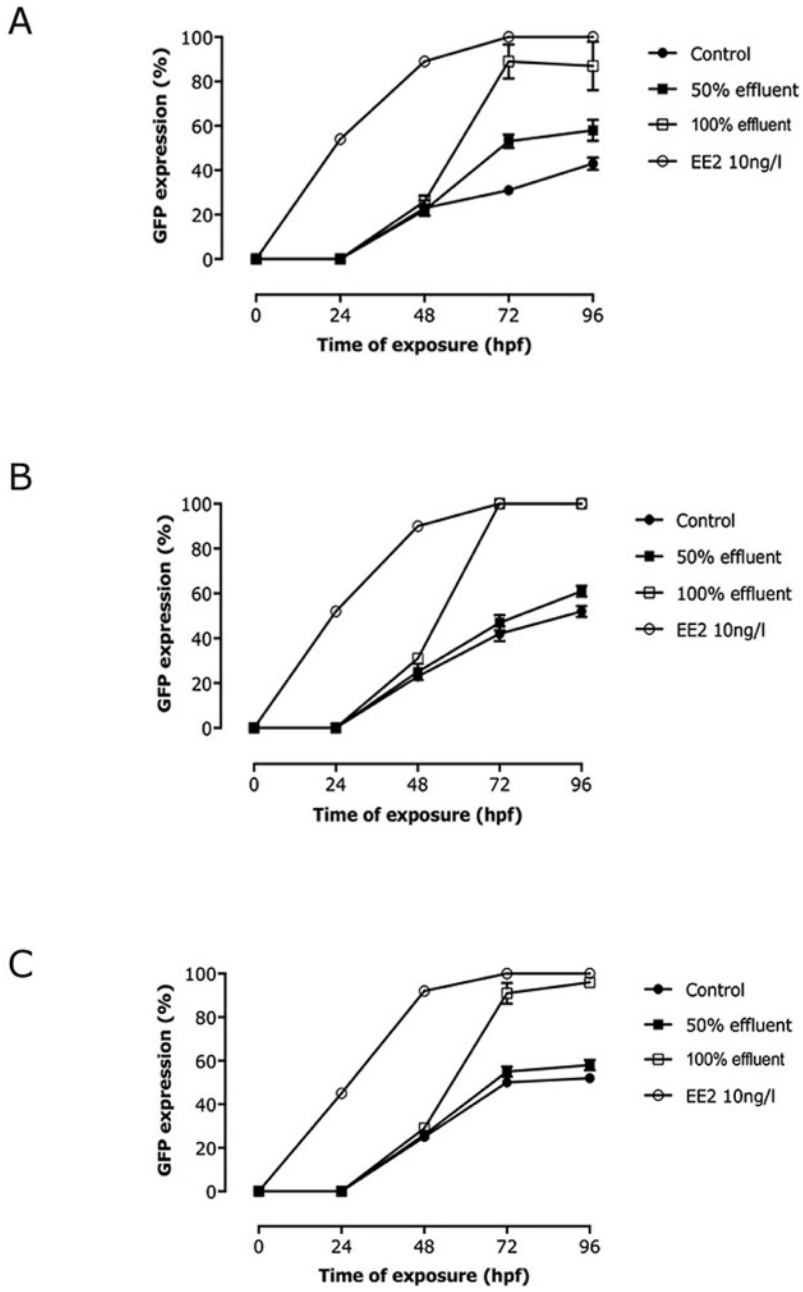

Fig. 7. Time related analysis of GFP expression found in the heart of ERE-GFP zebrafish embryos exposed to either a WwTW effluent or EE_2 from 0-96 hours post fertilisation. Data shows percentage of ERE-GFP embryos (*n*=45), expressing GFP in the heart for all exposure treatments for Exeter WwTW (**A**), Plymouth WwTW (**B**) and Tiverton WwTW (**C**). Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. Analysis was conducted three times for any given effluent collection. Data are reported as mean ± SEM.

Full details of time related analysis for all tissue responses are provided in the Supplementary Information (**SI T1**).

2.5 Discussion

2.5.1 Temporal variation of effluent oestrogenicity

Measurements of the steroidal oestrogens E_1 and E_2 confirmed the presence of oestrogens in each of the batches of effluents collected from the 3 WwTWs, but their concentrations (and the ratio between them) varied (more than 3-fold) over the 12-month period, supporting previous reports of the temporal variations in the oestrogenic content of wastewater effluents (Hemming *et al.,* 2004; Thorpe *et al.,* 2008; Vadja *et al.,* 2008; Zhou *et al.,* 2012). In particular, relatively high levels of E_1 (in comparison to E_2) were measured at all three treatment works (here ranging from 0.7-36.7 ng/L), and this is analogous with known concentrations commonly found in WwTW effluents (Vajda *et al.,* 2011; Furtula *et al.,* 2012b; Williams *et al.,* 2012). This can be explained by the overall slower degradation rate of E_1 and also by the rapid oxidation of E_2 to E_1 that occurs during wastewater treatment processes (Combalbert and Hernandez-Raquet, 2010; Desmiarti and Li, 2013). In comparison, the synthetic oestrogen EE_2 did not demonstrate any major temporal variability at any of the studied treatment works, with measured concentrations consistently ranging from between 0.4-0.7 ng/L, being similar to levels found in WwTW effluents in other studies (Cui *et al.,* 2006; Hannah *et al.,* 2009).

The efficiency of a WwTW in removing both natural and synthetic oestrogens is influenced by many different factors including temperature, rainfall and microbial activity, all of which can vary seasonally. Various studies have shown that higher temperatures in the summer months can enhance the efficiency of biological treatment processes in removing steroidal oestrogens, due to increased growth (and hence biodegradation) rates of microbial communities within wastewater treatment works, and the reverse is true during the winter (Nakada *et al.,* 2006; Jin *et al.,* 2008; Martinovic *et al.,* 2008). In this study, lower levels of steroid oestrogens were measured during the summer months (i.e. May to September) at all three treatment works, thus corroborating these findings. It should be noted however that high temperatures, in combination with lower rainfall, can result in microbial communities operating at a much lower efficiency rate (Cruikshank and Gilles, 2007).

Higher levels of rainfall, and hence greater dilution of WwTW effluents, can also lead to lower oestrogenic content, as has been illustrated in several studies (Kirk *et al.,* 2002; Diniz *et al.,* 2005; Phillips *et al.,* 2012). In this study we found lower levels of steroidal

oestrogens associated with higher rainfall levels when they occurred during the summer months. Contrasting with this however, high levels of rainfall (and hence higher flow rates of influent entering a WwTW) can also result in a reduction in total Hydraulic Retention Time (HRT), thus leading to reduced treatment efficiencies (Johnson *et al.,* 2005; Kuroda *et al.,* 2012; Phillips *et al.,* 2012; Kumar *et al.,* 2015). This may account for the extremely high EEQs observed at Plymouth WwTW during January 2014 (i.e. 85.6 ng/L^{-1} E2 EQs) when there had been a period of very high rainfall, combined with a relatively short HRT of only two and a half hours. A shorter HRT may also explain why this WwTW had the highest levels of oestrogens across the effleunts throughout the study periods. Other factors that may have contributed to the variability in the oestrogenic actvitiy found in the different WwTW effluents include fluctuations in the input of organic matter (quantified by biological oxygen demand [BOD] and total suspended solids [TSS]), that affect the binding of environmental oestrogens and thus their bioavailability for uptake.

Whilst seasonal influences such as temperature and rainfall are important factors in determining levels of steroidal oestrogens found in wastewater effluent, both the method of treatment employed at a WwTW and the influent composition are also extremely influential. Our study suggests that Exeter WwTW, with an Activated Sludge (AS) secondary treatment process, was more efficient at removing steroidal oestrogens compared with the Biological Filter (BF) processes in place at Plymouth and Tiverton WwTWs. This concurrs with other observatons on removal efficiency between the two processes (Johnson *et al.,* 2007). Exeter WwTW also has an HRT of eighteen hours compared with Plymouth and Tiverton (two and a half and twelve hours respectively), and this is also generally asscoiated with a more efficient removal of steroidal oestrogens (Johnson *et al.,* 2005; Huang *et al.,* 2008; Ejhed *et al.,* 2018).

Chemical makeup of the wastewater influent to a treatment works can also affect the subsequent effluent quality. In this study, the majority of wastewater entering all three WwTWs was predominantly domestic, with only a small percentage of industrial input (between 3.3 to 7.1%). We found no link between the total population equivalent size of a particular WwTW and the amount of oestrogenic activity in the resulting effluent; as an example, EEQs at Exeter and Tiverton were found to be very similar (23.15 and 24.17 ng/L^{-1} respectively), whilst their p.e. values were considerably different (141698 and 23648 respectively). However, some of the seasonal variations in the oestrogen

content of the WwTW effluents at Exeter and Plymouth may be due to changes in the seasonal resident population (e.g. due to student enrolment for local universities) and which may contribute to the higher oestrogenic content observed at both works during March 2013 and January 2014.

The high variability in the oestrogenic content of the WwTW effluents emphasise the importance of avoiding extrapolations from single time point analysis. This can easily produce misleading results including on the potential for biological effects; spikes in oestrogen levels can relatively be easily missed that may induce significant biological impacts (Panter *et al.,* 2000; Diamond *et al.,* 2005). Investigations into the effects of oestrogens on cancerous cells, have shown intermittant exposures can be unexpectedly potent (Ponnusamy *et al.,* 2017). Consideration of the variable nature of the exposure may thus be important when assessing effluent oestrogenicity.

2.5.2 Biological responses in the ERE-GFP zebrafish model

For EE_2 there was no significant effects on survivorship for any of the exposures and this is concordant with several other studies in the literature for this/similar EE_2 exposure concentrations (Versonnen and Janssen, 2004; Schäfers *et al.,* 2007; Jackson and Klerks, 2019).

A pronounced mortality for exposures to 100% effluent during March 2013 and Jan 2014 was observed at Plymouth WwTW, which correlated well with increases in the oestrogenic potency of the wastewater effluent at these particular time-points. Identification of the physiological mechanisms behind this reduced survival was beyond the scope of this study but some associations between the seen effect and measured chemicals in the effluent may be inferred, based on previous findings in the literature. For example, increased mortality may be due to an inability to metabolise oestrogen-induced proteins, such as VTG, which was found to be significantly elevated in embryos for both time periods in this study. This can result in an accumulation of VTG in the kidneys and subsequent renal failure and loss of homeostasis and has been linked to several environmental oestrogens commonly found in wastewater effluent such as EE_2, NP and E_2 (Folmar *et al.,* 2001b; Lange *et al.,* 2001; Magliulo *et al.,* 2002). In this study, the individual levels of the steroidal oestrogens present in the wastewater effluent are unlikely to have caused higher levels of VTG induction *per se*, however, in combination

their effect can be additive (Silva *et al.,* 2002; Brian *et al.,* 2005), therefore producing an EEQ high enough to account for the observed response.

It is worth noting that physiochemical parameters related to effluent quality at Plymouth WwTW, in this instance elevated levels of both chloride and ammonia (up to 4000 and 40 mg/L respectively) caused by saline infiltration from the local sewerage system and its discharge location on the Plymouth Sound, may have contributed to the observed increases in mortality found at this treatment works. Several studies have shown that ammonia can have detrimental effects on fish survival by causing asphyxiation or renal failure or by affecting the immune system (Constable *et al.,* 2003, Camargo and Álonso, 2006; Da Silva *et al.,* 2009).

It is important also to consider that the wastewater effluents used in this study would contain a mixture of many different chemicals, including polycyclic hydrocarbons (PAHs) and heavy metals (e.g., tributyltin and lead), any of which may have contributed towards the observed mortality in fish. For example, as heavy metals have a propensity to bind to phosphates and other organic molecules, including base residues of DNA, they can cause genotoxic effects such as DNA mutations by altering the primary and secondary structures of DNA (Ferraro *et al.,* 2004).

With regards to hatching rates, reduced successes for the EE_2 and effluent exposures are also in concordance with other studies (Smolders *et al.,* 2002**;** Versonnen and Janssen, 2004), but again the mechanism for this are not known. It is feasible that the observed delay in hatching seen in this study is a result of a lack of proteolytic enzymes, which are known to be required for chorion-softening and subsequent hatching in teleost fish (Kim *et al.,* 2006; Sano *et al.,* 2008), due to possible disruptions in the regulation of the hatching gene tetraspanin cd63 (Trikic *et al.,* 2011). Recently, De la Paz *et al.,* (2017), demonstrated that exposure to the selective estrogen receptor modulator (SERM) triazole, lead to a strong inhibition of hatching in zebrafish embryos. This was found to correlate with a decrease in the secretion of hatching gland cells, which are known to express all three ER subtypes during early zebrafish life stages (Chandrasekar *et al.,* 2010; Hao *et al.,* 2013).

2.5.3 GFP expression

Tissue-specific effects in response to the different oestrogenic effluents were observed in the ERE-GFP zebrafish model, further substantiating its good sensitivity and

applicability for use in environmentally relevant toxicological research and chemical testing and screening. Recently this ERE-GFP transgenic model has been developed further in a pigment free (Casper) background that will potentially allow for more effective analysis of responses in body tissues and for juvenile/adult life stages (Green *et al.,* 2016).

As expected, exposure to EE_2 induced responses in a very wide range of tissues, including heart, liver, somite muscle and forebrain, again in agreement with other studies (Gorelick *et al.,* 2011; Lee *et al.,* 2012; Green *et al.,* 2016) and is similar to GFP tissue expression patterns induced by E_2, highlighting the potential for a wide range of biological effects following exposure to this synthetic oestrogen. This is of concern, as whilst concentrations of EE_2 found in wastewater effluent are generally lower than endogenous steroidal oestrogens, its higher potency in fish (10-50-fold higher than that of E_1 and E_2, *in vivo*) and greater resistance to breakdown in the aquatic environment (Jurgens *et al.,* 2002; Thorpe *et al.,* 2003) potentially make EE_2 a major contributor to reproductive disruption in wild fish. In addition, several studies have shown a tendency of EE_2 to bio-accumulate at environmentally relevant concentrations in whole body tissues and the bile of fish (Al-Ansari *et al.,* 2010; Fenlon *et al.,* 2010; Yang *et al.,* 2014).

Expression of GFP in the ERE-GFP transgenic zebrafish was observed in the heart, liver, muscle somites and otic vesicle, with varying sensitivities, for all WwTW effluents and this correlated well with the varying concentrations of steroidal oestrogens found in the different wastewaters throughout the study periods. Other tissues, such as the fore-brain and neuromasts, responded only to certain effluents (namely Plymouth WwTW). These observed differences in tissue response patterns are consistent with results from previous studies with our ERE-GFP zebrafish model (Lee *et al.,* 2012), and with other researchers using GFP transgenic fish exposed to oestrogenic chemicals (Gorelick *et al.,* 2011; Brion *et al.,* 2012) and likely reflect tissue specific responses to different oestrogenic chemicals. The pattern of tissues responses in the Plymouth WwTW in January 2014 were most likely a consequence of exposure to high levels of E_2 (where there was strong GFP induction in the liver). In contrast, in the Tiverton WwTW exposure in April 2014, there was little or no GFP expression in the liver, but induction was found in the somites, suggesting a response mediated by a non-steroidal oestrogen, with possible chemical candidates for this such as nonylphenol or BPA (Lee *et al.,* 2012). The different tissue specific responses also indicate the potential for differing health

effects even for a single WwTW effluent over time/season. The differences in tissue specificities for the GFP induction may be due to differences in tissue expression and interactions with the three oestrogen receptor subtypes (ERα, ERβ1 and ERβ2) and other related receptors that can form heterodimers with ERs. The ERE-GFP model however is designed to capture responses to all ER subtypes, and thus from these studies the specific receptor pathways for the tissue effects seen here cannot be determined. Future work using RNA sequencing techniques could help to identify and quantify differentially expressed genes and establish pathways and processes affected in these target tissues for selected oestrogens.

In this study, a high sensitivity of the heart to both EE_2 and 100% WwTW effluent exposures was observed, which is consistent with responses seen in another ERE transgenic zebrafish line (Gorelick *et al.,* 2014). In addition, and unique to this tissue, significant responses in GFP were also observed in fish exposed to 50% effluent further substantiating the sensitivity of the heart to environmental oestrogens and supporting a crucial role of oestrogenic signalling in heart development (Moreman *et al.,* 2017).

In general, there was a high concordance between all measured GFP expression responses in the zebrafish model for the detection of steroidal oestrogens from the same environmental samples and for EE_2. In terms of sensitivity, the ability of the model to allow for the analysis of individual tissues, such as the heart or brain, through non-destructive fluorescence microscopy, further demonstrates its advantages over other quantification methods such as Western Blot or qPCR.
In addition, this study reveals an ability to visualise a MOI in a live organism, through the binding of a chemical and subsequent activation of the ERs, demonstrating the potential of the ERE-GFP zebrafish model in terms of defining AOPs for an oestrogenic chemical. For example, the KE of increased *vtg* production is described in this study that could be used to evidence exposure to an oestrogenic chemical operating through the AOP network.

2.5.4 VTG expression

Significant *vtg* mRNA induction was detected in the ERE-GFP fish for exposure to all three WwTW effluents, mirroring the higher concentrations of steroidal oestrogens measured, suggesting that the levels of E_1 and E_2 present alone were capable of inducing the observed *vtg* responses. This is in agreement with the general concensus

that steroidal oestrogens are largely responsible for *vtg* induction in fish exposed to wastewater effluents, contributing to most of the oestrogenic activity (up to 80%), together with EE_2 (Desbrow *et al.*, 1998; Rodgers-Gray *et al.,* 2000). To further evaluate the ERE-GFP zebrafish as a method for assessing the health effects of an oestrogenic WwTW effluent, this study sought to establish how GFP responses in the model compared in terms of sensitivity for the detection of oestrogens with VTG induction. A positive correlation between *gfp* mRNA induction and *vtg* mRNA induction was observed in EE_2 exposures and this was also the case for WwTW effluent exposures (albeit not as significant), showing a clear relationship between the two, which is to be expected given the major role of estrogen in VTG production (Hara *et al.,* 2016). These data suggest that the transgenic zebrafish model is as responsive a biomarker system for the detection of oestrogens as VTG induction. Direct comparison of *gfp* with *vtg* mRNA expression demonstrated a greater sensitivity for the latter, notably in terms of the magnitude of responses. This might be expected considering the huge induction capacity for VTG (and its protein product) of up to a million-fold (Tyler *et al.,* 1996) and may reflect differences in the dynamics of synthesis and turnover in mRNA between the two genes. A greater scope for the magnitude of change for VTG also means that it is more likely to see changes/induction with a statistical significance compared to *gfp* induction which was found to be more variable response.

2.5.5 Conclusion

This study has demonstrated that the ERE-GFP transgenic zebrafish model can be applied to detect environmental oestrogens in real world wastewater effluent samples and exhibits tissue-specific effects similar to those seen for exposure to different individual environmental oestrogens. Differences in GFP tissue patterning (and of other biological endpoints) were found to be reflective of the varying concentrations of steroidal oestrogens found in the different wastewaters, (both temporally and between treatment works), and illustrated the considerable dynamic nature of the oestrogenic potencies on the WwTW effluents. These findings further highlighting the importance of integrating biomonitoring approaches to complement analytical chemistry techniques in the monitoring of estrogenic chemicals in wastewater effluents.

Whilst these results suggest that GFP induction is less sensitive as a detection method than for VTG induction for detecting responses to oestrogen, the ERE-GFP zebrafish provides an integrative model system to help understand more fully the tissues targeted for more effective health effects analyses, and thus ecological risk assessment posed by wastewater effluents, specifically in respect of AOP frameworks.

2.6 References

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2.9 Supplementary Information

SI Table 1 Time related tissue analysis of GFP expression found in ERE-GFP zebrafish embryos exposed to either a control (unexposed), WwTW effluent or EE2 from 0-96 hours post fertilisation. Data shows percentage of ERE-GFP embryos (n=45), expressing GFP in the heart, liver, brain, somites and otic vesicle for all exposure treatments for Exeter WwTW, Plymouth WwTW and Tiverton WwTW. Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. Analysis was conducted three times for any given effluent collection. Data are reported as mean ± SEM.

SI Fig 1. **3ERE-Gal4ff/UAS-GFP system in zebrafish.** The ERE-Gal4ff transgene contains three synthetic EREs, one TATA, a Gal4ff reporter, and two *Tol2* elements; and the UAS-GFP transgene contains five tandem repeats of the UAS-Gal4 binding sequence, an E1B minimal promoter, and an EGFP reporter. An oestrogenic signal is detected when the chemical–ER complex binds to the ERE, which activates Gal4ff; subsequently, Gal4ff protein binds to UAS to induce EGFP.

SI 3 Protocol description of LC-MS used for chemical detection of the steroidal oestrogens in the WwTW effluent.

SI Fig 2. **Expression of GFP in different tissues in whole ERE-GFP zebrafish embryos exposed to various wastewater effluents for 96 hours post fertilisation.** Data shows the percentage of zebrafish larvae with observed GFP expression in the heart, liver, otic vesicle, brain and somite muscles for unexposed, EE_2 (10 ng/L, positive control) and wastewater effluent (50% and 100%) exposed treatment groups at Exeter WwTw (**A**), Plymouth WwTw (**B**) and Tiverton WwTw (**C**). Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period.

SI Fig 3. Seasonal variation in GFP expression in the liver, heart and somite muscle in whole ERE-TG zebrafish embryos exposed to selected wastewater effluents from fertilisation to 96 hours post fertilisation. Transgenic zebrafish embryos in control (unexposed) (**A**), EE_2 (10 ng/L, positive control) (**B**), and wastewater effluent (100%) exposed groups at Exeter WwTw (**C-F**), Tiverton WwTw (**G-J**) and

Plymouth WwTw (**K-N**). Effluent exposures were conducted using 24-hour composite wastewater effluent samples collected at 4-time points over a 12-month period. Analysis was conducted three times for any given effluent collection. GFP expression is shown in lateral view in the liver (i) and somite muscle (iii), and in ventral view in the heart (ii). Bars = 100 μm.

SI Fig 4. Quantification of GFP induction, measured by Western blotting, in whole ERE-TG zebrafish embryos in control (unexposed) embryos or in embryos exposed to EE_2 (10 ng/l) or a graded effluent concentration (50% or 100%). Results show exposures conducted every 3/4 months over a 12-month period at Exeter (A-D), Plymouth (E-H) or Tiverton (I-L) WwTW. The intensity of GFP was analysed using Image J (http://rsbweb.nih.gov/ij/), normalised to the intensity of the alpha-tubulin band and shown as a fold increase in GFP over the level in control zebrafish embryos. Data is reported as mean of 3 replicates ± SEM. Asterisks denote a significant difference (*p<0.05, **p<0.01) compared with control.

SI Fig 5. Levels of whole body induction of GFP mRNA (A) and VTG mRNA (B) in ERE-GFP transgenic zebrafish (at 96 hours post fertilisation) against the measured oestrogenic content of the WwTW effluents – Data is expressed as oestradiol-17b equivalents (E_2 EQs; calculated based on the measured concentrations for three target steroid oestrogens - EE_2, E_2 and E_1, multiplied by their relative potencies compared to E_2 and summed using concentration additivity.

Site	Exeter Countess Wear WwTW				Plymouth Central WwTW				Tiverton Allers WwTW			
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**
Control	% 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
50% effluent												
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
100% effluent												
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
EE2												
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

SI Table 1

SI 2 Water Chemistry LC-MS Quantification

Ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-26 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) maintained at 25 °C. Injection volume was 5 µL and mobile phase solvents were water (A) and acetonitrile (B) in an initial ratio (A:B) of 70:30. Separation was achieved at 25 °C using a flow rate of 0.25 mL/min with the following gradient: 70:30 to 30:70 in 8 min; 30:70 to 0:100 and held for 4 min; return to initial condition for 12 min and equilibration for 6 min. MS/MS was performed in the Multiple Reaction Mode (MRM) using ESI in the negative mode, and one characteristic fragment of the deprotonated molecular ion [M−H]− was used for quantitation. Other parameters were optimised as follows: capillary voltage −3.3 kV, extractor voltage 8 V, multiplier voltage 650 V, source temperature 120 ◦C, desolvation temperature 300 ◦C. Argon was used as collision 3 gas (P collision cell: 3×10−3 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 the oestrogen 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. Five point calibration curve (R2 > 0.99) covered the range 125–2500 pg (injected on column) for all compounds, within the linear range of the instrument.

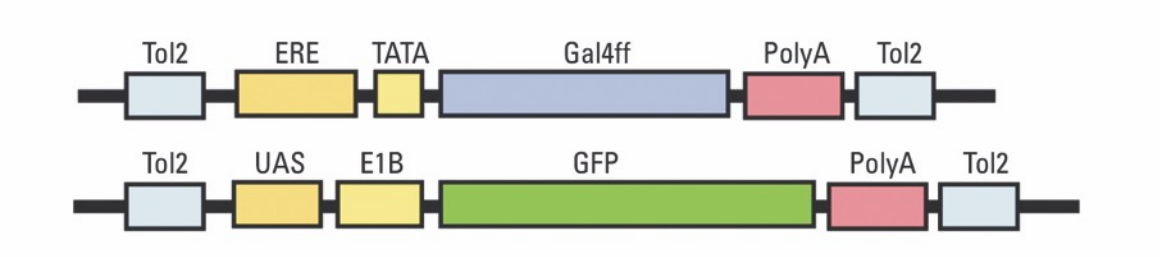
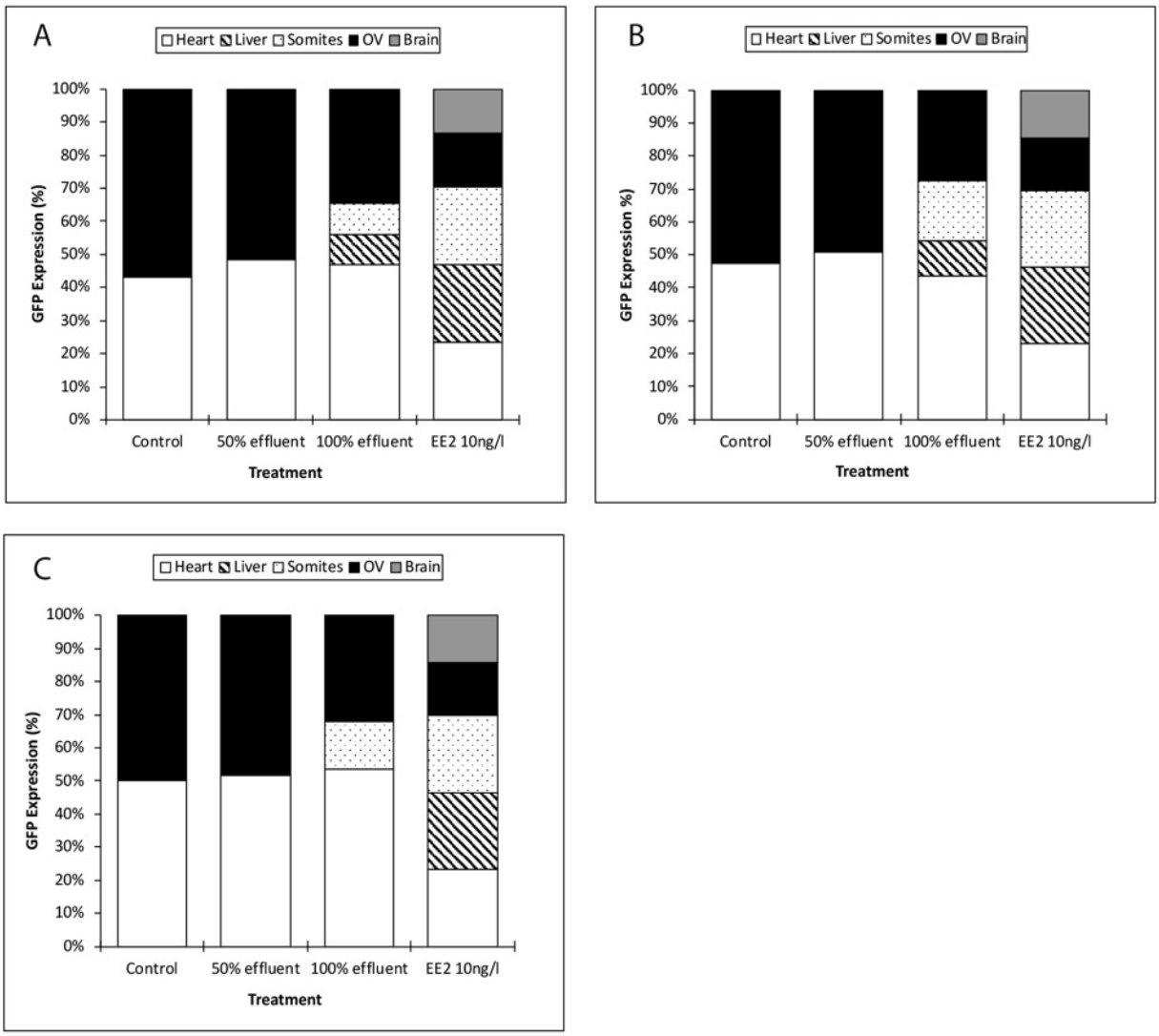
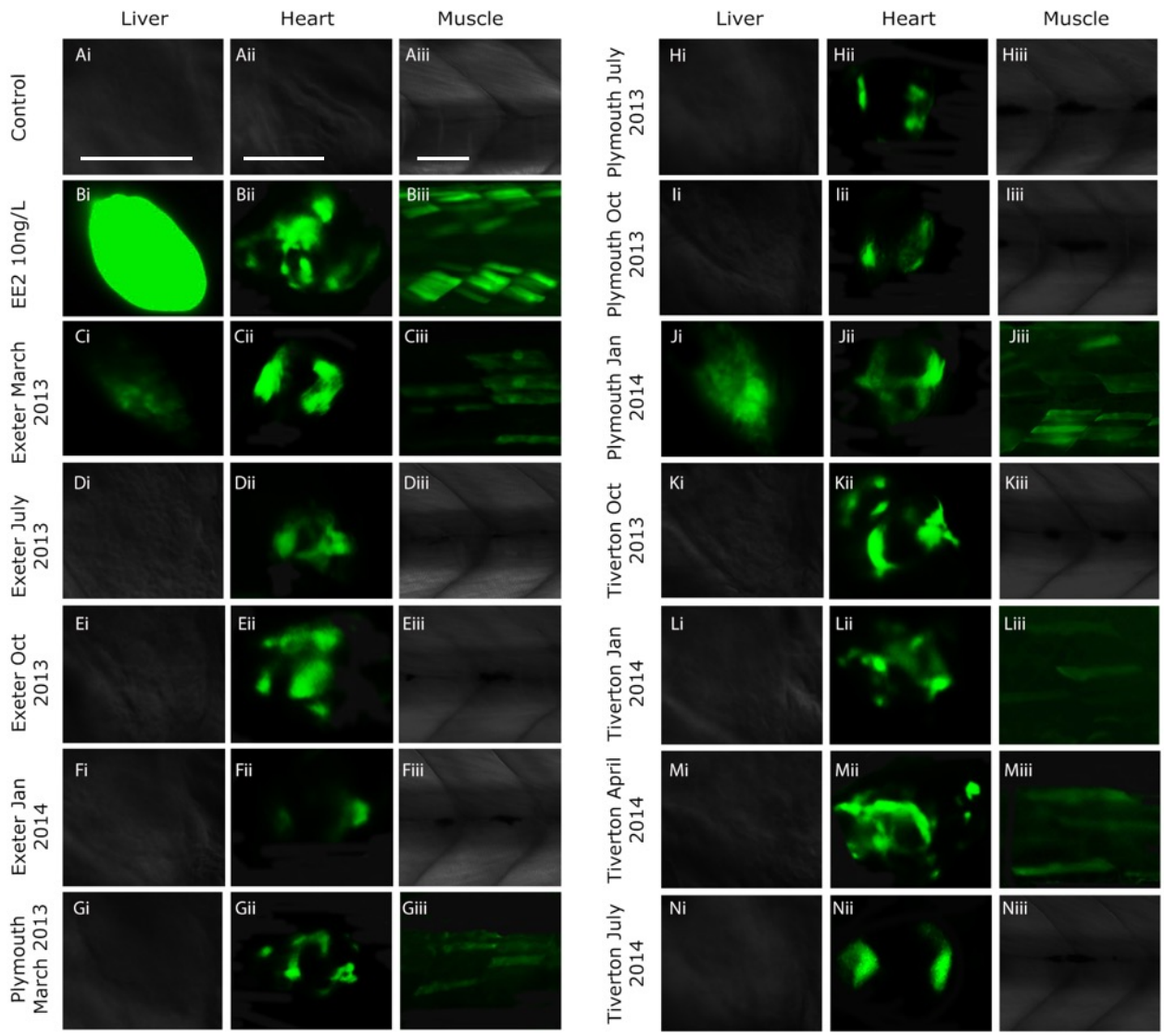


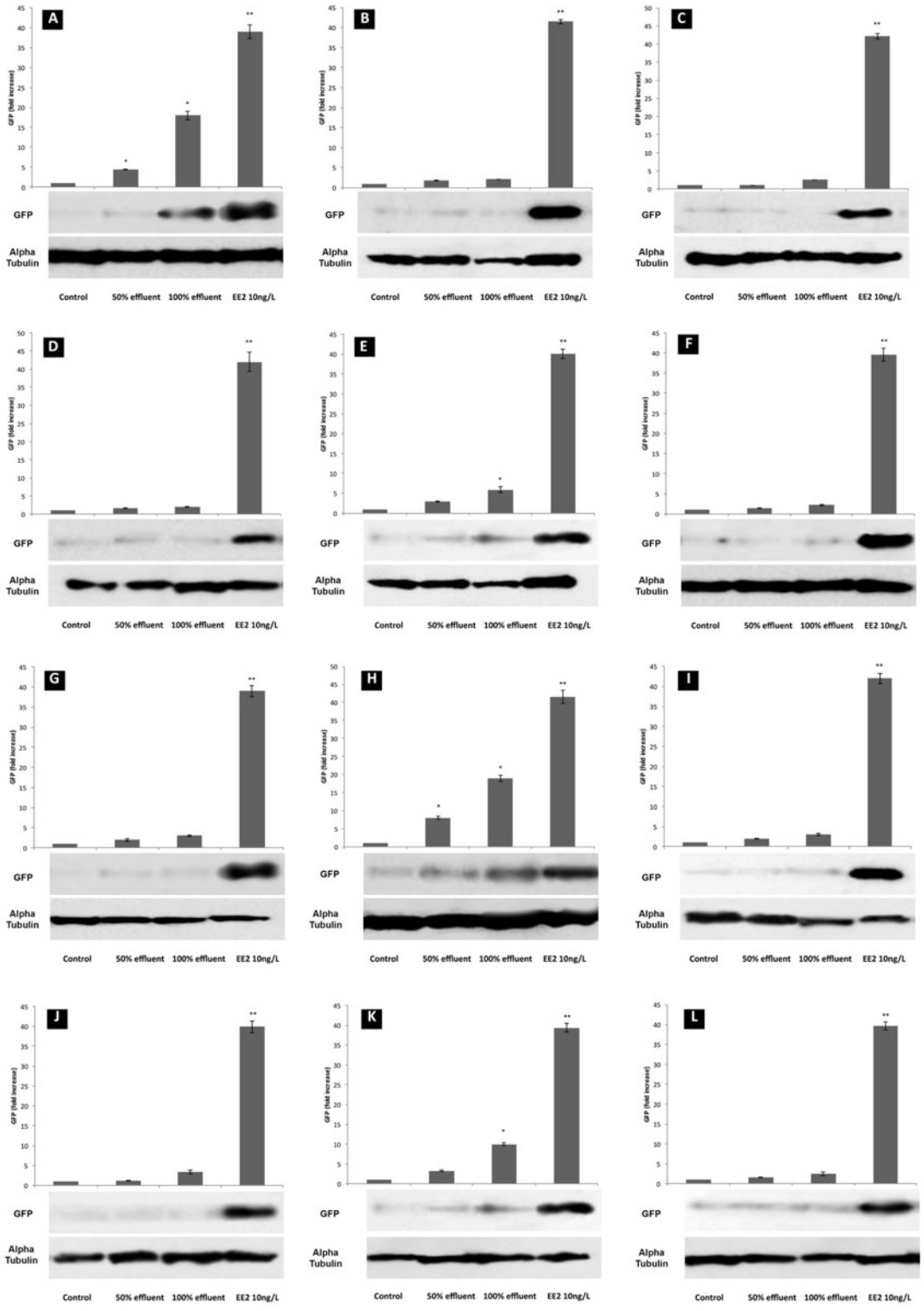
Figure S1.


A
Heart Liver Somites OV Brain
GFP Expression (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Control
50% effluent
100% effluent
EE2 10ng/l
Treatment
B
Heart Liver Somites OV Brain
GFP Expression %)
Control
50% effluent
100% effluent
EE2 10ng/l
Treatment
C
Heart Liver Somites OV Brain
GFP Expression (%)
Control
50% effluent
100% effluent
EE2 10ng/l
Treatment

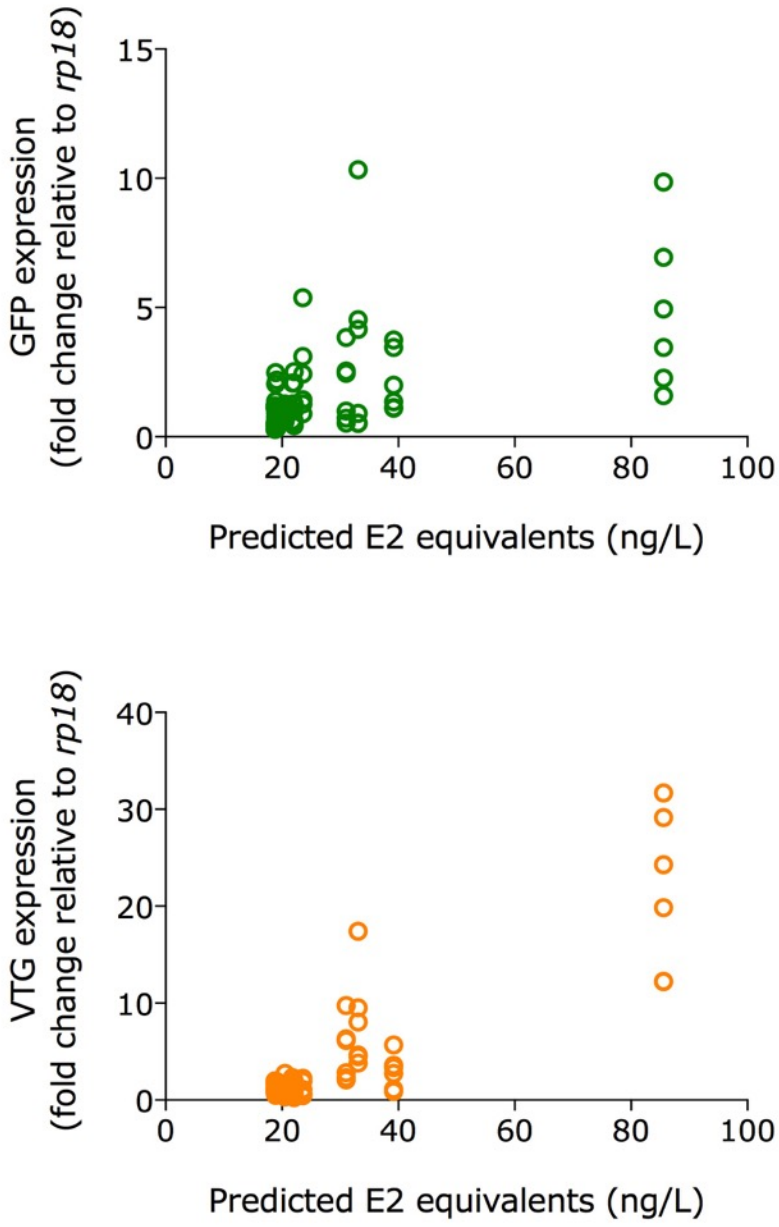
SI Fig. 2


Liver
Heart
Muscle
Control
Ai
Aii
Aiii
EE2 10ng/L
Bi
Bii
Biii
Exeter March 2013
Ci
Cii
Ciii
Exeter July 2013
Di
Dii
Diii
Exeter Oct 2013
Ei
Eii
Eiii
Exeter Jan 2014
Fi
Fii
Fiii
Plymouth March 2013
Gi
Gii
Giii
Liver
Heart
Muscle
Plymouth July 2013
Hi
Hii
Hiii
Plymouth Oct 2013
Ii
Iii
Iiii
Plymouth Jan 2014
Ji
Jii
Jiii
Tiverton Oct 2013
Ki
Kii
Kiii
Tiverton Jan 2014
Li
Lii
Liii
Tiverton April 2014
Mi
Mii
Miii
Tiverton July 2014
Ni
Nii
Niii

SI Fig. 3


A
B
C
D
E
F
G
H
I
J
K
L
GFP (fold increase)
GFP
Alpha Tubulin
Control
50% effluent
100% effluent
EE2 10ng/L

SI Fig. 4


GFP expression
(fold change relative to rp18)
15
10
5
0
0
20
40
60
80
100
Predicted E2 equivalents (ng/L)
VTG expression
(fold change relative to rp18)
40
30
20
10
0
0
20
40
60
80
100
Predicted E2 equivalents (ng/L)

SI Fig. 5

Chapter 3.

An integrative approach using an ERE-GFP transgenic zebrafish model to assess chronic health effects and life stage sensitivities for exposure to an estrogenic wastewater treatment works effluent.

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3.1 Abstract

A wide range of health effects in fish have been reported for exposure to wastewater treatment work (WwTW) effluents ranging from overt toxicity for effluents from poorly treated influent to feminised responses in males commonly seen even for effluents where tertiary treatments of influents has occurred.

In this study, a novel oestrogen-responsive green fluorescent protein (ERE-GFP) Casper transgenic zebrafish model was applied to quantify for chronic health effects, life stage sensitivities and for oestrogenic biological activity in an oestrogenic WwTW effluent. Zebrafish were exposed to either a WwTW effluent at 100% or 50% dilution or a reference oestrogen positive control (17α-ethinyl-estradiol [EE_2]) during the following developmental life periods: (i) 0-90 days (ii) 21-60 days (iii) 60-90 days. Measured endpoints included survival/hatching rate, somatic growth, gonadal development, GFP induction (measured in target tissues such as the liver via fluorescence microscopy, and via Western blotting or mRNA induction in whole embryos) and *vtg* mRNA induction.

In wastewater effluent exposures, biological effects such as increased mortality (observed in early life stage fish [48 hours post fertilisation]), decreased hatching rates and up-regulation of *vtg* mRNA were observed in chronic exposures, with the latter also seen in 21-60 and 60-90 day exposure periods. Differing patterns of GFP expression, in terms of target tissues and during different developmental life stages were observed in chronically exposed zebrafish. Recorded LOECs for *vtg* and *gfp* mRNA induction were 19.35 and 42.64 ng/L^{-1} EEQs respectively, demonstrating a higher magnitude for responses in *vtg* induction. However, when compared, induction levels for *vtg* and *gfp* mRNA were much more closely linked, with differing degrees of strength dependent on the developmental life stage. Exposure to the wastewater effluent (and to 10 ng EE_2) was also found to delay the maturation of testis in male fish, with the opposite effect in females, i.e. accelerated ovary development, whilst exposure to both 50% and full strength WwTW effluent (and to 10ng EE_2 /L^{-1}) resulted in skewed sex ratios in favour of females.

This study demonstrates how an integrated approach, supplementing analytical chemistry with a biosensor transgenic zebrafish and effect-based analysis, can be effective for providing information on the potential for both chronic and acute

health effects for a WwTW effluent, using an adverse outcome pathway framework and further highlights the importance of an organism's development stage and sex with regards to potential health effects associated with oestrogen exposure.

3.2 Introduction

It is now widely accepted that various chemicals entering the environment via WwTW effluents can have endocrine disrupting effects in aquatic organisms, and notably in fish. Endocrine related effects in fish associated with WwTW exposure include the induction of vitellogenin (VTG; an oestrogen dependent yolk precursor), intersexuality (Bahamonde *et al.,* 2015a/b; Vajda *et al.,* 2008; Williams *et al.,* 2009), alterations in blood hormone levels (Hoger *et al.,* 2006, Jeffries *et al.,* 2008; Sepulveda *et al.,* 2004) and impaired sexual development and reproduction (Jobling *et al.,* 2006; Kidd *et al.,* 2007; Nash *et al.,* 2004; Thorpe *et al.,* 2007).

Evidence from both *in vivo* and *in vitro* studies suggests that both endogenous and synthetic oestrogens, including oestrone (E1), 17β-estradiol (E_2) and 17α-ethynyloestradiol (EE_2), a component of the contraceptive pill, which are responsible for the majority of oestrogenic activity observed in wastewater effluents and their receiving waters (Nakada *et al.,* 2004, Nelson *et al.,* 2007; Ting *et al.,* 2017) and some oestrogen-mimicking chemicals, such as 4-nonylphenol (NP) and bisphenol-A (BPA), are responsible for the WwTW exposure effects on reproductive development and function in fish (Careghini *et al.,* 2015; Press-Kristensen *et al.,* 2015). Exposures to oestrogenic chemicals have also been linked to adverse reproductive health effects in humans, including reduced sperm count/quality, disruptions in the development of the reproductive tract and oestrogen dependent cancers (Zhao and Li, 2015; Talsness, 2009) as well as wider health effects on the immune system, obesity and cardiovascular disease (Hatch *et al.,* 2010; Lang *et al.,* 2008; Sweeny 2009).

In the UK it has been reported that around one third of rivers in England may contain levels of environmental oestrogens sufficient to cause disruption to reproduction in fish (Williams *et al.,* 2009). This is highly variable however, and oestrogenic discharges into these rivers from WwTWs is dependent on a variety of factors, including the type of wastewater treatment process and the operational conditions in those WwTWs, which can affect chemical removal efficiencies and hence the overall oestrogenicity of an effluent (Liu *et al.,* 2009), and the level of effluent dilution in the river. Seasonal factors, particularly temperature, can also impact of rates of chemical biodegradation (Johnson *et*

al., 2009; Cooper *et al.,* in press). As a consequence, exposure levels to environmental oestrogens can vary over time and may occur intermittently, rather than continuously. Intermittent exposures to certain oestrogens however, has been reported to be equally impactful on fish health as for continuous chronic exposures (Diamond *et al.,* 2005; Panter *et al.,* 2000).

Although concentrations of oestrogenic chemicals typically found in WwTW effluents and surface waters are generally low (in the ng to ug/L range depending on the specific chemical) many have been shown to bio-concentrate in aquatic organisms (Da Silva *et al.,* 2013; Fenlon *et al.,* 2010; Yang *et al.,* 2014) and this may increase their biological effects. This is supported for findings with EE_2 where chronic exposures can result in reproductive failure that does not occur for short term exposures to the same concentrations (as evidenced by Nash *et al.,* 2004 and Parrott and Blunt, 2005). Further studies on EE_2 have also evidenced direct population-level consequences (Kidd *et al.,* 2007, Schwindt *et al.,* 2014) and indirect effects on food webs for chronic exposures (Kidd et al., 2014). Moreover, endocrine disrupting chemicals (EDCs), including oestrogens, that act through a similar or common mode of action can have additive effects on the reproductive physiology of fish adding further to the debate over health risks and current recommended exposure thresholds (Brian *et al.,* 2007; Rajapakse *et al.,* 2002**;** Relyea and Diecks, 2008; Silva *et al.,* 2002).

Differences in sensitivity across the developmental life stages of an organism exposed to an oestrogenic effluent can be a critical factor in determining the effect induced and severity of the effect. Many studies have shown that early life stages (i.e. embryonic development) can be especially vulnerable to chemical effects and these exposures can furthermore result in effects that manifest in later life stages (Coe *et al.,* 2010; Länge, *et al.,* 2009; Schwindt *et al.,* 2014). Increasingly, there is evidence also for the epigenetic effects of oestrogenic chemicals (Bouwmeester *et al.,* 2016; Liu *et al.,* 2016 Strömqvist *et al.,* 2010) which involves heritable alterations that are not due to changes in the DNA sequence. Discharge of WwTW effluents and the chemicals they contain may therefore have greater implications for fish populations than are currently accounted for.

Due to the complexity and dynamic nature of real-world exposures, traditional 'chemical-by chemical' approaches are now often viewed by many as too simplistic as they may underestimate the potential health impacts of chemical pollutants in aquatic environments. In addition, differences between the *in vitro* potency of individual oestrogens can be significantly different from *in vivo* potencies, with several studies suggesting that some *in vitro* assays may underestimate the oestrogenic potential of wastewater effluent (Huggett *et al.,* 2003; Wehmas *et al.,* 2010). As such, there is a drive to apply more holistic and integrated approaches for assessing chemical effects on animal health, and biosensor transgenic fish show great promise as effect-based monitoring tools in both mixture and temporal effects analyses (Brion *et al.,* 2012; Gorelick *et al.,* 2014; Li *et al.,* 2017). Furthermore, there is the potential for facilitating the advancement of environmental risk assessment founded on the adverse outcome pathway (AOP) framework by providing evidence for a 'molecular initiating event' (MIE) i.e. a direct interaction of a chemical with its molecular target, and subsequent 'key events' (KE) in the toxicity pathway (at the cellular and organ level), leading to an adverse outcome (AO) at the organism and population level (Ankley *et al.,* 2010).

In previous work an oestrogen responsive element (ERE) – Green Fluorescent Protein (GFP) transgenic zebrafish model in a pigment free (Casper) strain of zebrafish (ERE-GFP-Casper; Green *et al.,* 2016) was developed and applied to quantify responses to individual oestrogens and identify their target tissues (Green *et al.,* 2016). In this study, the health effects and life stage sensitivities following chronic and acute exposures to a WwTW effluent with known (measured) oestrogenic content were investigated using the ERE-GFP-Casper model. The responses measured included assessments on survivorship, growth, sex and sexual development (via gonadal histopathology), fluorescence induction in both target tissues (via fluorescence microscopy) and in whole bodies via quantification of GFP mRNA (via qRT-PCR). Levels of whole body/liver GFP mRNA were also compared with levels of vitellogenin mRNA, a well-established biomarker for oestrogen exposure.

3.3 Materials and methods

3.3.1 Test animals and husbandry.

A transgenic zebrafish strain developed at the University of Exeter, ERE-GFP Casper (3×ERE:Gal4ff and UAS:GFP) was employed in this work. The ERE-GFP Casper line was derived from crossing an established transgenic ERE-GFP line (Lee *et al.,* 2012) with a Casper strain acquired from University College London (**SI 1**). This line contains silenced *roy* (dark) and *nacre* (silver) pigmentation genes, resulting in highly translucent skin (White *et al.,* 2008). All zebrafish were raised at 28.5+/-1 ^{0}C C under a 12-h light, 12-h dark cycle photoperiod regime, fed to satiation three times daily with ZM Fry food 000/100 (Zebrafish Management Ltd., Wincester, U.K.) and Liquifry No. 1 (Interpet, Dorking, U.K.) up to 21 dpf, progressing to live newly hatched Brine Shrimp (*Artemia*) and frozen Gamma Omega 3 Enriched Brineshrimp (supplied by Zebrafish Management Ltd., Wincester, UK) from 21 dpf to adulthood.

3.3.2 Water supply.

The supply of water to the laboratory dosing system was maintained using reverse-osmosis with the addition of salts (as described in OECD Guideline 203). Water temperature and pH levels were monitored daily and ranged between 26.5 and 27.6 ◦C in all experiments, with pH levels between 6.8 and 7.7. Dissolved oxygen concentrations were maintained at >80% of the air saturation throughout all exposures. Conductivity ranged between 200 and 260 µS/cm.

Dilution water and test chemical flow-rates were measured twice weekly; flow-rates to each individual aquaria were set at 20 mL/min (working volume of 20 L) to provide a 75% replacement every 24 hours.

3.3.3 Experimental design.

ERE-TG GFP Casper zebra fish (*n* = 180 per treatment x 3 replicate 20L glass tanks[1]) were exposed to either a dilution water control (DWC), a reference oestrogen positive control (EE_2 10 ng/L^{-1}) or a wastewater effluent at a

1 Prior to 30dpf all zebrafish were housed in smaller nursery tanks (5L) placed within the larger 20L tanks.

concentration of either 100% or 50% during the following developmental life periods: (i) 0-90 days (ii) 21-60 days (iii) 60-90 days (**Fig. 1**). These life stages were chosen to cover the entire life to sexual maturation (i), the period of sexual differentiation in zebrafish (ii), and the period of gametogenesis (sexual maturation, iii). The 50% effluent concentration was obtained by diluting the 100% 1:1 with tap water. Solvent free stock solutions of EE_2 (≥ 98% purity, purchased from Sigma, Poole, UK) were prepared every three days by adding 250ul of a concentrated stock solution of EE_2 (prepared in HPLC grade acetone, purchased from Fisher Scientific) to a 2.5L sterile glass Winchester bottle, previously acid washed and rinsed with acetone (Analytical reagent grade, purchased from Fisher Scientific) and dichloromethane (HPLC grade, purchased from Fisher Scientific) to remove any traces of contaminants. After evaporation of the acetone, at room temperature, 2.5 L of dilution water was added, and the solution stirred for approximately 2 hours. The solvent free stock was then delivered to another glass mixing vessel via a peristaltic pump, to mix with the dilution water for the final working concentration of 10 ng/L.

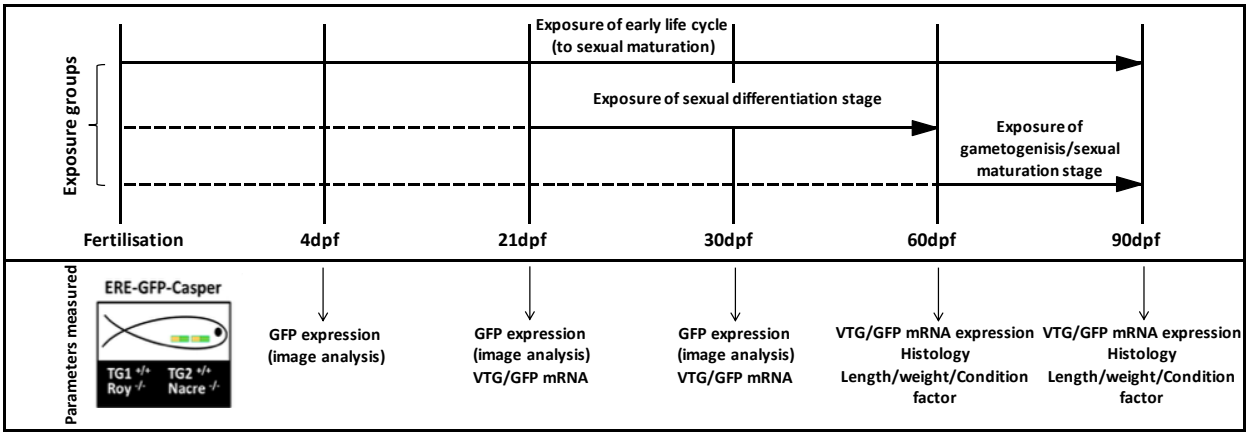

Figure 1. Design of the zebrafish effluent exposure experiment. The times different endpoints were measured are indicated by arrows. Exposures were conducted to a dilution water control (DWC), EE_2 (10ng/l) or a 100% or 50% effluent concentration for the different developmental life stages indicated. There were three replicate tanks per treatment.

3.3.4 Wastewater treatment works effluent.

Effluent was collected at twice weekly intervals in batches of 500 L over a period of 13 weeks using a Glass Reinforced Plastic (GRP) water tank. Each consignment of effluent was collected from the final effluent stream at Exeter WwTW between the hours of 08.00 and 09.00 a.m., immediately transported

back to the university and then placed into a fully enclosed stainless steel holding tank chilled to around 4 ^{o}C, before being transferred via a peristaltic pump system into glass mixing tanks, where it was slowly acclimated to the desired test temperature of 28 ^{o}C before being pumped into the relevant test exposure tanks (**SI 2**).

As with most WwTWs found in the UK, Exeter receives waste primarily from domestic sources, with some industrial/trade waste. Details of the treatment processes and the types of industrial/trade discharges for Exeter WwTW are listed in the supplementary information (**SI 3).** The total population equivalent (P.E.) or unit per capita loading for this treatment works was 141698. P.E. is a value for quantifying the organic strength of wastewater discharges and reflecting the net waste of the surrounding human population and industrial facilities and is based on a biological oxygen demand (BOD) of the water of 60 g per person/per day. P.E. has been used also to predict/model steroid oestrogen content within WwTWs. The total and industrial P.E. for Exeter WwTW was obtained from the water utilities responsible for its operation, i.e. South West Water. Dissolved oxygen concentrations for all consignments of effluent were above 80%, with pH values ranging between 6.5 and 7.2, and temperatures ranging from 17.3 to 20.7 ^{o}C.

3.3.5 Estimation of the relative potencies of E_1, E_2, and EE_2.

The relative potencies of $1E_2$:0.75E1:$40EE_2$ was used to calculate the equivalent concentrations of E_2 for comparing with the quantification of GFP/VTG mRNAs. These equivalency potencies were chosen based assessments reported by Van den Belt *et al.* (2004) and Jobling *et al.,* (2006) for the induction of VTG in zebrafish (*Danio rerio*). This toxic equivalency approach is recommended for the risk assessment of oestrogenic chemical mixtures, such as wastewater effluent, and is based on the assumption that the combined effects of endogenous oestrogens are essentially additive.

3.3.6 Fish sampling and analysis of phenotypic endpoints.

For this study triplicate tanks of fish were sampled at various time periods during development (as shown in Fig. 1) and examined for GFP expression using image analysis (*n*= 15 fish per treatment) VTG/GFP mRNA expression

(*n*= 15 fish per treatment) and histological analysis (*n*= 30 fish per treatment). Survival, total fork length and wet weight (to the nearest 1mm and 0.01 g respectively) and condition factor (*K*-factor) were also recorded for each fish. The *K*-factor was calculated for each fish using the formula: *K*-factor = (weight (g)/length $(cm)^3$) × 100. Other measured endpoints included hatching rate success (at 72 & 96 hpf). Fish were sacrificed by terminal anesthesia with benzocaine (0.5 g/L; ethyl-*p*-aminobenzoate; Sigma, Poole, U.K.) All animal use and protocols were carried out ethically in accordance with U.K. Home Office guidelines (Animals (Scientific Procedures) Act 1986).

3.3.7 Analytical measurement of concentrations of environmental oestrogens; Estrone (E_1), 17β-estradiol (E_2), 17α-ethinyl-estradiol (EE_2)).

Three 2.5 L effluent samples were spiked immediately upon collection with a 5% methanol/ 1% acetic acid mix and stored at 4 ^{o}C prior to Solid Phase Extraction (SPE). 1L samples of these effluents were then were spiked with an internal standard (0.05% of an estrogenic mixture containing E_1d4, E_2d4 and C13NP at 2 µg/ml, i.e. 100 ng total and EE_2D4 at 0.5 µg/ml, i.e. 10 ng) and filtered using glass wool and filter paper to remove any large particulates, prior to extraction onto pre-conditioned (with HPLC-grade methanol and water) 1 g Oasis HLB cartridges. The cartridges were then eluted with 20 mL 95:5 methanol:water, rinsed with HPLC water to remove any salts and then evaporated to dryness under vacuum for subsequent measurement of E_1, E_2, and EE_2 (via UHPLC-MS/MS). All positive controls (EE_2) and dilution water controls were treated and extracted under the same conditions. Ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analyses was carried out using a Waters Acquity UHPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). The limits of detection were 0.35, 0.25, and <0.5 ng/L for E_2, E_1, and EE_2, respectively. Details of the LC-MS Method can be found in Supplementary Information (**SI 4**).

3.3.8 Image analysis.

Fluorescence microscopy (Zeiss Axio Observer.Z1; Zeiss, Cambridge, UK) was used to visualise GFP expression in our zebrafish using the same parameters

with a 1-10x objective (for the different life stages), employing the Axiovision digital software programme for the production of images. Exposure times employed were kept consistent and were dependent on the area photographed due to the varying levels of fluorescence intensity seen in the different target tissues, (50 ms for head region, 20 ms for mid trunk region, and 400 ms for tail section). GFP expression was measured at 4, 21 and 30 dpf, respectively (*n*= 15 per treatment). Prior to live imaging all fish were anesthetized with 0.4% tricaine, mounted in 4% methylcellulose in a glass-bottom dish and oriented in dorsal, ventral and lateral views. All images were aligned, and the contrast adjusted using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA) keeping the same intensity for all samples.

3.3.9 Histological analysis.

All samples were fixed *in toto* in Bouin's fixative (Fisher Scientific) for a maximum of 6 hours and washed twice in 70% ethanol (at 12 and 24 hours post sampling) and subsequently processed for 16 hours in a semi-enclosed tissue processor (Shandon Citadel 2000). In brief, tissue samples were progressively dehydrated through a series of graded industrial methylated spirits (IMS) solutions (70-100%), washed in ethanol and Histoclear and then embedded in paraffin wax at 60^{o}C. A minimum of 30 zebrafish per treatment were then serially sectioned into 5-µm sections on a rotary microtome (Leica model). For each fish, a minimum of 6 transverse serial sections were obtained (taken 50-µm and 250-µm apart along the gonad for males and females, respectively) mounted onto glass slides and subsequently stained with hematoxylin- eosin, before examination using a Zeiss Axioshop 40 light microscope (magnification 10-100x) to determine both gonadal sex and maturity and to assess for any alterations in the structural organization of the gonad, i.e. intersex. The stage of gonadal maturity (**SI 5a**) was recorded according to the standardised criteria described in the OECD Histopathology Guidance document (Johnson *et al.,* 2009) and using a numerical staging system (**SI 5b**).

3.3.10 RNA extraction and cDNA synthesis.

For VTG/GFP mRNA expression analysis all gonadal and whole-body samples were immediately snap-frozen in liquid nitrogen upon sampling and stored at

−80 °C until use. Whole-body homogenates for 30 and 60dpf fish were prepared in liquid nitrogen prior to RNA extraction. Total RNA was extracted from all samples using Trizol Reagent (Sigma), according to the manufacturer's instructions. The amount of RNA was quantified using a NanoDrop 1000 spectrophotometer and RNA purity determined through the measurement of the A260/A280 and A260/230 ratios respectively. Reverse transcription was subsequently carried out by incubating 1 ug RQ1 DNase treated (Promega) total RNA with 5 mM random hexamers (Eurofins MWG Operon) 10 mM dNTPs and 1ul MMLV-Reverse transcriptase (Promega) in the appropriate buffer for 60 min at 37 ^{o}C and 10 min at 42 ^{o}C. Polymerase chain reaction (PCR) was performed in an iCycler hermocycler coupled to a MyiQ detector (Bio-Rad. Hercules, CA, USA).

3.3.11 Real-time quantitative PCR.

RT-qPCR was carried out on cDNA samples for each treatment group and sampling point (*n*= 8 for each treatment) for selected target genes with the iCycler iQ Real-time Detection System (Bio-Rad Laboratories Inc., CA, USA) using the following primers:

Vtg1 (fw) 5'-GCCAAGAAAGAACCCAAACTG-3',
Vtg1(rev) 5'-GAGATGAGAGCCACTGAAG-3';
EGFP(fw) 5'-CGACGGCAACTACAAGAC3',
EGFP(rev) 5'TAGTTGTACTCCAGCTTGTGC -3'.

Primer sequences were either designed with Beacon Designer software Premier Biosoft International, Palo Alto, USA) or derived from previously published information (Brion *et al.,* 2012), and obtained from MWG-Biotech (Ebersburg, Germany). In brief, primer pairs were optimized for annealing temperature (Ta), specificity confirmed by melt curve analysis, and the detection range, linearity and amplification efficiency (*E*) established using serial dilutions of zebrafish cDNA. RT-qPCR was carried out using Absolute QPCR SYBR Green Fluorescein mix (ABgene), with an initial activation step of 95 °C for 15 min followed by 40 cycles of denaturation (95 °C, 10 s) and annealing (appropriate Ta, 45 s) and final melt curve analysis. Expression levels of ribosomal protein L8 *(rpl8)* were used as a 'housekeeping' gene to normalize the expression of other genes using a development of efficiency correlated relative quantification as described previously (Filby and Tyler, 2005).

3.3.12 Statistical analysis.

In all cases, data were checked for normality using the D'Agostino-Pearson test and for homogeneity of variance using Bartlett's or Levene's test prior to statistical analysis. Data meeting these tests were analysed using parametric tests, e.g. analysis of variance (ANOVA) procedures, whilst data that did not conform to normality were subject to nonparametric tests, e.g. Kruskal–Wallis. Post hoc analysis was performed against controls using the Dunnett's test and with the Tukey test for between-treatment comparisons. Data were transformed, where necessary, using log10. Any significant differences from expected sex ratios or stages of gonadal development were tested using the chi-square test. All statistical analyses were run in GraphPad Prism 7 (GraphPad Software, Inc., San Diego, USA). Statistical significance was accepted as $p < 0.05$ for all comparisons and values are quoted as mean values and ranges as standard error of the mean.

3.4 Results

3.4.1 Concentrations of steroid oestrogens in the exposure effluent.

Analytical measurements for E_1, E_2 and EE_2 (the three principal oestrogens predominately found in WwTw effluent) were used to characterise the oestrogenic content of the effluent. Measured concentrations of E_1 from the different consignments of effluent collected from Exeter WwTW ranged from between 0.2 to 4.8 ng/L during June 2014 (mean 1.3±0.98 ng/L) up to between 2.7 and 39.6 ng/L during Sept 2014, (mean 20.0±5.1 ng/L), with E_2 concentrations varying between 0.4 and 46.4 ng/L over the experimental period (**Table 1**). Calculated equivalent concentrations (using $E2_{equiv/VTG;GFP}$ =1E2 + 40 EE_2 + 0.75E1) showed that the oestrogenic activity of the effluent ranged from 19.35 ng EEQs/L to 42.64 ng EEQs/L (**SI 7**).

Physiochemical parameter	Jun-14	Jul-14	Aug-14	Sep-14
pH	7.0 ± 0.05	6.8 ± 0.05	7.2 ± 0.05	6.8 ± 0.10
Rainfall (mm)	8.0 ± 1.4	5.5 ± 3.4	7.0 ± 0.8	2.3 ± 1.2
Temperature (^oC)	20.1 ± 1.43	20.7 ± 0.87	19.3 ± 0.61	17.3 ± 0.43
Suspended solids mg/l	13 ± 0.64	14 ± 1.36	7 ± 1.15	12 ± 0.94
BOD mg/l	7 ± 0.76	5 ± 0.22	<3.5 ± 0.08	3 ± 0.65
COD mg/l	23 ± 0.32	40 ± 0.45	35 ± 0.62	68 ± 0.82
NH3 mg/l	4.6 ± 0.12	6.2 ± 0.06	<0.2 ± 0.01	4.7 ± 0.03
Cl- mg/l	n/a	n/a	n/a	n/a
E1 ng/l	1.3 ± 0.98	0.5 ± 0.13	2.0 ± 1.0	20.0 ± 5.1
E2 ng/l	0.6 ± 0.18	1.0 ± 0.35	0.5 ± 0.14	9.6 ± 4.91
EE2 ng/l	0.5 ± 0.03	0.5 ± 0.03	0.5 ± 0.03	0.5 ± 0.04

Table 1. Measured physiochemical parameters of the treated wastewater effluent collected from the WwTW at Exeter, during all experimental periods. Data are reported as mean ± SEM.

Measured concentrations of EE_2 in the positive control tanks were between 70% and 120% of nominal concentrations (mean 8.6±3.6 ng/L). Concentration of EE_2, E_2 and E_1 in all control tank water were below the minimum detection level i.e. 0.5; 0.4; 0.2 ng/L, respectively, and were ascribed as zero.

3.4.2 Phenotypic endpoints

3.4.2.1 Survival and hatching success for 0-90-day exposures

There was no significant effect on survival rates in 24 hpf embryos, with the exception of EE_2 treatments (460/540=85%, p=<0.001, **Fig 2A**). At 48 hpf, survival rates were significantly different compared with untreated controls in embryos exposed to 100% effluent and EE_2 treatments (493/540 = 91%, p=<0.05, 434/540 = 80%, p=<0.001, respectively, **Fig 2A**). At 96 hpf, the number of surviving embryos was 78% in EE_2 (10 ng/L) treatment (419/540). There were significant differences in survival rates between both 50% and 100% effluent and EE_2 treated embryos at all time-points (p=<0.05). All surviving embryos in control groups had hatched by 72 hpf. The earliest observed hatching occurred at 48 hpf in the 100% WwTW effluent exposures (82/493=17%, p=<0.001). There was a significant difference in hatching rates at 72hpf for embryos exposed to 100% effluent compared with EE_2 (317/493 = 64%, p =<0.0001, 358/419 = 85%, p=<0.01, respectively, **Fig 2B**). At 96 hpf, hatching rates were significantly different compared with untreated controls in embryos exposed to 100% effluent and EE_2 treatments (445/493 = 90%, p =<0.001, 385/419 = 92%, p=<0.0001, respectively, **Fig 2B**). Post-hatch survival in each treatment group was >80%, - exceeding OECD guidelines of ≥70% survival for controls (OECD, 2011), and with no statistically significant differences between treatments (data not shown).

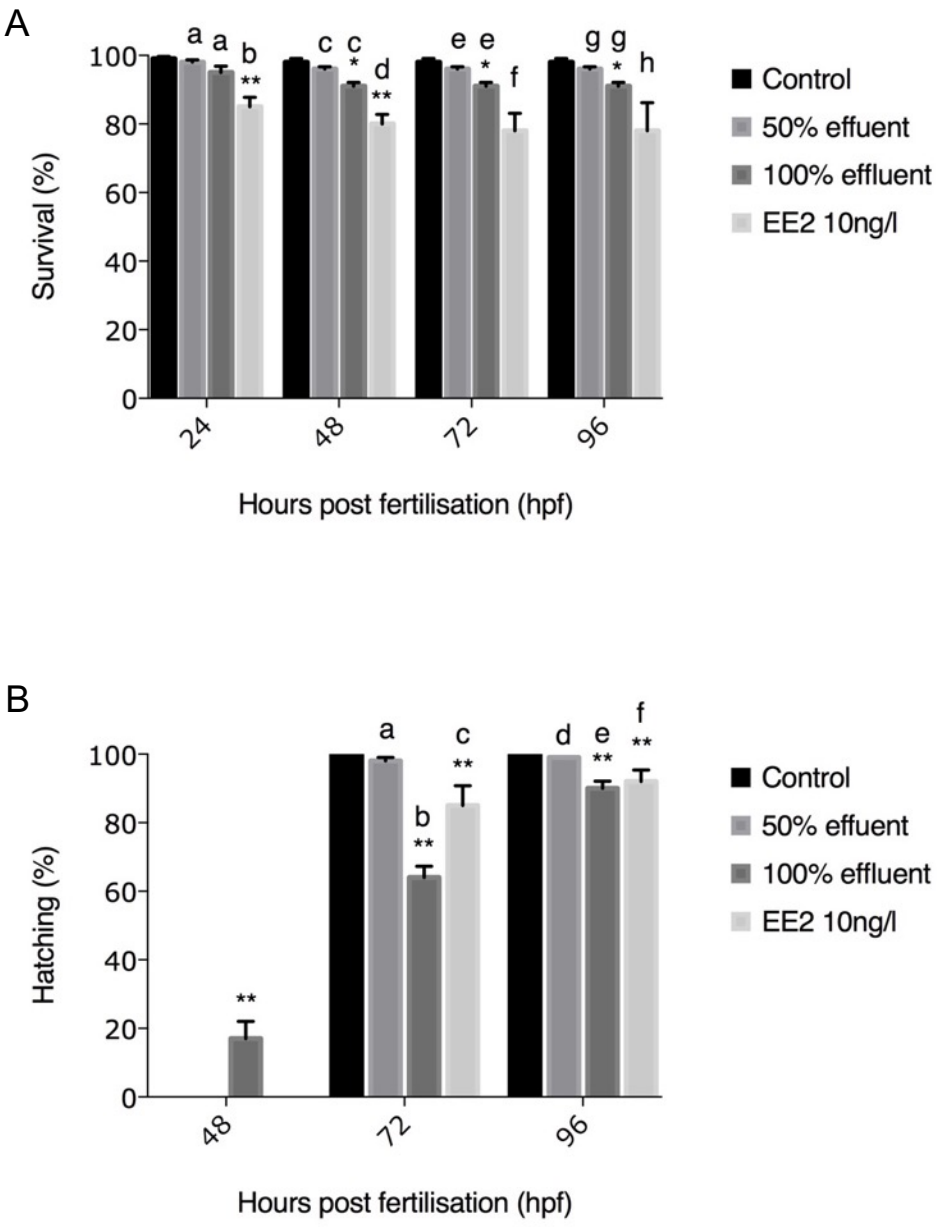

Figure 2. Hatchability rates (B) and subsequent survival (A) to 96 hours post fertilisation in ERE-GFP zebrafish embryos. Data shows transgenic zebrafish embryos in control (unexposed) EE_2 (10 ng/L, positive control) or exposed to a WwTW effluent concentration (50% or 100%). Data are reported as mean ± SEM. Asterisks denote a significant difference (*p<0.05, **p<0.01) compared with control.

3.4.2.2 Survival rates for exposures between 21-60 days and 60-90 days

Survival in each treatment group for these exposure periods was >80% and there were no statistically significant differences between treatments (data not shown).

3.4.2.3 Body size/condition factor

Male and female body weight, length and condition factor were measured at 60dpf and 90dpf for all developmental exposures (see Table 2)

- Males

In the 0-90dpf continuous exposure the length and weight of male ERE-TG Casper zebrafish at 60dpf was significantly higher in the 100% effluent exposure groups compared to untreated controls (p =<0.01), whilst length and weight in those fish exposed to EE_2 was significantly decreased (p =<0.01). There was no observed effect in 50% effluent exposure groups. No significant differences in *K*-factor were found in any of the treatment groups. At 90 dpf, the response pattern for weight and length were consistent for 100% effluent and EE_2 exposure groups as at 60 dpf. Fish in the 50% effluent at 90dpf were larger than in controls (p =<0.05). At 90 dpf there was no significant difference in *K*-factor between any of the exposure groups. For the exposure during the period of sexual differentiation (21-60dpf), males were of a greater length in 100% effluent treatments compared to untreated controls (p =<0.05). In the EE_2 treated fish both length and weight were significantly lower (p =<0.01), with no observable effect in 50% effluent exposure groups. Significant differences in *K*-factor (lower compared with controls) were observed in both 100% effluent and EE_2 treated fish (p =<0.01) but not in 50% effluent exposures. For exposures carried out during the period of gametogenesis and sexual maturation (60-90 dpf) males were smaller in length and weight in 100% effluent exposure (p =<0.01), and of a lower length for exposure to 50% effluent and EE_2 (p =<0.01). Significant differences in *K*-factor (higher when compared to controls) were observed in males exposed to 100% effluent and EE_2 (p =<0.01).

- Females

In chronic exposures the length and weight of females at 60 dpf was significantly higher in 100% effluent exposure groups compared to untreated controls (p =<0.01), whilst length and weight in those fish exposed to EE_2 was significantly lower (p =<0.01). Females exposed to 50% effluent were significantly heavier than untreated controls (p =<0.05). At 90 dpf, length and weights in female fish were higher for exposure to both 50% and 100% effluent (p =<0.05) compared with controls. Females exposed during the period of

sexual differentiation (21-60 dpf), were larger in length and weight (at 60 dpf), in 100% effluent treatments (p =<0.01) compared with controls. Females exposed to 50% effluent were greater in length only compared with controls (p =<0.01). Again, the overall length and weight in those fish exposed to EE_2 was significantly lower (p =<0.05). As occurred in males, *K*-factor in females were significantly lower in both 100% effluent and EE_2 treated fish (p =<0.01) but not in 50% effluent exposures. For exposures carried out during the period of gametogenesis and sexual maturation (60-90 dpf) females in both 100% effluent and EE_2 treatments were smaller in size - length and weight (p =<0.01), and females exposed to 50% effluent were smaller in length only (p =<0.01).

Window of exposure	Treatment	Length (mm)		Weight (g)		*K*-factor	
		Female	Male	Female	Male	Female	Male
0-90 days – 60dpf	Control	27±0.5	23±0.6	0.3±0.01	0.2±0.01	1.8±0.08	1.8±0.1
	50% effluent	27±0.5	25±0.6	0.4±0.03*	0.3±0.01	1.8±0.1	1.6±0.06
	100% effluent	29±0.4**	27±0.7**	0.5±0.02**	0.4±0.02**	1.8±0.06	1.6±0.09
	EE2 (10 ng/l)	24±0.5**	19±1.1**	0.2±0.01**	0.1±0.03**	1.6±0.03	1.8±0.08
0-90 days – 90dpf	Control	36 ±1.6	35 ±0.5	0.6± 0.02	0.4± 0.01	1.5± 0.3	1.0± 0.04
	50% effluent	38 ±1.2*	37 ±0.6	0.7± 0.04*	0.5± 0.02*	1.1± 0.06	0.9± 0.02
	100% effluent	40 ±1.1*	38 ±0.7**	0.7± 0.02*	0.5± 0.03*	1.2± 0.08	0.9± 0.03
	EE2 (10 ng/l)	33 ±1.0**	30 ±0.7**	0.5± 0.04**	0.3± 0.02	1.5± 0.1	1.0± 0.03
21-60 days – 60dpf	Control	27±0.5	24±0.6	0.4±0.01	0.3±0.01	1.8±0.08	1.8±0.1
	50% effluent	30±0.2**	25±0.5	0.4±0.02	0.3±0.02	1.7±0.09	1.7±0.05
	100% effluent	33±0.3**	26±0.7*	0.5±0.01*	0.2±0.1	1.0±0.03**	1.4±0.09**
	EE2 (10 ng/l)	26±0.4*	21±0.4**	0.3±0.01*	0.2±0.01*	1.2±0.04**	1.2±0.07**
60-90 days – 90dpf	Control	36±0.5	35±0.3	0.6±0.01	0.4±0.01	1.5±0.0	1.0±0.03
	50% effluent	33±0.6*	30±0.5*	0.5±0.03	0.3±0.01	2.0±0.1	1.5±0.08
	100% effluent	31±0.5**	29±0.5**	0.4±0.03*	0.3±0.02**	1.8±0.1*	1.4±0.08*
	EE2 (10 ng/l)	28±0.8**	29±0.8**	0.4±0.04**	0.4±0.02	1.9±0.09	1.9±0.07**

Significantly different from untreated controls * p<0.05, ** p<0.01

Table 2. Length, weight, condition factor (*K*-factor) determined in adult male and female ERE-GFP Casper zebrafish in control (unexposed) fish or in fish exposed to EE_2 (10 ng/l) or a graded WwTW effluent concentration (50% or 100%) across different developmental stages. Data are reported as mean ± SEM. Asterisks denote a significant difference (*p<0.05, **p<0.01) compared with untreated controls.

3.4.2.4 Sex ratios

In chronic exposures tanks, there was sex ratio bias in favour of males in the control population of around 2:1 male to female ratio and this was also the case for the 50% effluent exposures (**Fig. 3A**). In contrast, for exposed to 100% effluent or EE_2 the observed sex ratios were 1:3 male to female $(\chi^2)_{(1, N =120)}$ =9.64, p< 0.001) and 1:4 male to female $(\chi^2)_{(1, N =120)}$ =14.04, p< 0.001)

respectively at 60 dpf. A proportion of fish in EE_2 exposure groups (7%) had not undergone sexual differentiation (and/or the sex could not be identified). This response pattern was similar at 90 dpf (2:1 male to female ratio in controls and 50% effluent exposures and 1:3 male to female ratio in 100% effluent exposures, $(\chi^2)_{(1, N =144)} = 12.59$, $p<0.001$). The female skew however was greater for the EE_2 exposure, and showed an enhanced effect compared with at 60dpf (1:11 male to female ratio, $(\chi^2)_{(1, N =144)} = 26.13$, $p<0.0001$, **Fig. 3B**). For the groups exposed during the period of sexual differentiation (21-60 dpf) sex ratios at 60 dpf in both control groups and in fish exposed to 50% effluent were as observed for the 60 dpf assessments for the 0-90 dpf exposures, i.e. a 2:1 male to female ratio (**Fig. 3C**). A shift in sex ratio was observed in fish exposed to both 100% effluent $(\chi^2)_{(1, N =90)} = 7.52$, $p< 0.001$) and EE_2 $(\chi^2)_{(1, N =90)} = 11.38$, $p< 0.0001$), with a 4:7 male to female ratio and 7:15 male to female ratio, respectively (**Fig. 3C**). For fish exposed during gametogenesis to sexual maturation (60-90 dpf) at 90 dpf the sex ratio in control groups was consistent with the other control groups, i.e. 2:1 male to female ratio. In the 50% effluent exposure there was 3:2 male to female ratio (**Fig. 3D**) and in the 100% effluent $(\chi^2)_{(1, N =144)} = 12.59$, $p< 0.01$) and EE_2 $(\chi^2)_{(1, N =144)} = 26.13$, $p< 0.0001$) the observed ratios were 5:4 male to female and 6:7 male to female, respectively.

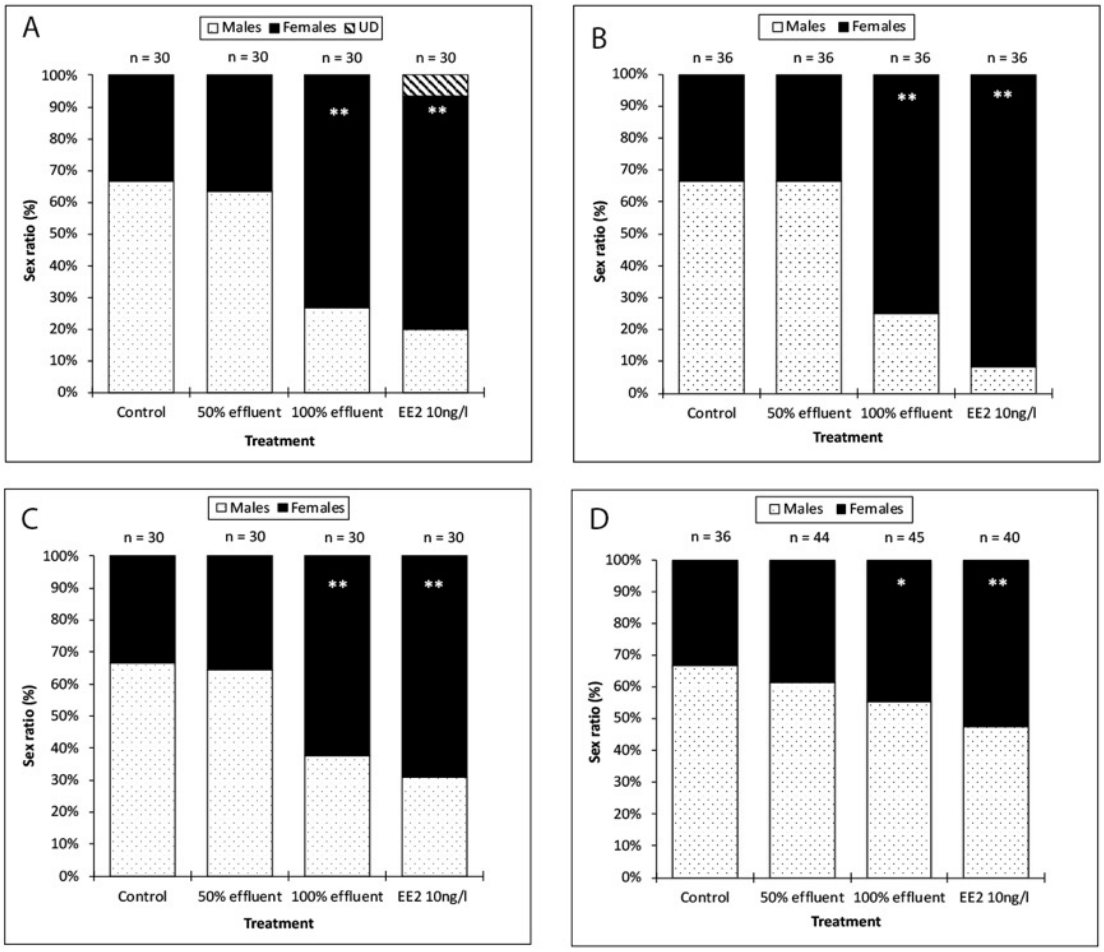

Figure 3. Sex ratios in ERE-GFP Casper zebrafish. Data shows transgenic zebrafish at 60dpf or 90dpf in control (unexposed) fish or in fish exposed to EE_2 (10 ng/l) or a graded WwTW effluent concentration during the following developmental periods: (A) 0-90 days (60dpf); (B) 0-90 days (90dpf) (C) 21-60 days (60 dpf) and (D) 60-90 days (90 dpf). Data are reported as mean ± SEM. Asterisks denote statistically significant differences (*p<0.05, **p<0.01) from expected sex/maturity index ratios according to a chi-square contingency table.

3.4.2.5 Sexual differentiation and development

Chronic exposure to either 100% effluent $(\chi^2)_{(2, N=28)} = 12.2$, $p < 0.001$) or EE_2 $(\chi^2)_{(2, N=26)} = 16.8$, $p < 0.0001$) appeared to delay testis maturation; at 60 dpf 55% of control males were at stage 3 but none were beyond stage 2 in the 100% effluent and EE_2 exposure groups (**Fig. 4Ai**). At 90 dpf there were, however, no significant effect on testis development in the 50% or 100% effluent exposed males compared with controls. In contrast at 90 dpf, males testis development in EE_2 exposures was still delayed with none reaching stage 3 $(\chi^2)_{(2, N=26)} = 6.9$, $p < 0.05$) (**Fig. 4Bi**).

For fish exposed during the period of sexual differentiation (21-60 dpf) there was no significant effect on testis development at 60dpf in any of the treatment groups (**Fig. 4Ci**). Exposure during gametogenesis to sexual maturation (60-90 dpf), to 100% effluent however resulted in a delayed testis development in males $(\chi^2)_{(2, N=37)} = 6.3$, $p < 0.05$), where only 15% of males were classed being as stage 3 versus 40% in the respective controls (**Fig. 4Di**). Intersex gonads (i.e., testis-ova) were not observed at any of the experimental time points.

In contrast with males, in female fish sampled at 60 dpf in the 0-90 dpf exposure, the effects of EE_2 appeared to enhance ovary maturation $(\chi^2)_{(2, N=38)} = 3.1$, $p < 0.0001$), with 21% of fish classed as stage 3 and none had advanced beyond stage 2 in the respective controls (**Fig. 4Aii**). There was a tendency – non significant - for this to be the case in females exposed to 100% effluent also (14% at stage 3). At 90dpf, females in 100% effluent exposure groups $(\chi^2)_{(2, N=39)} = 14.3$, $p < 0.0001$) had a higher ovary maturity index compared to control groups, with 22% classed as at stage 4 in the effluent group compared with 0% in the controls (**Fig. 4Bii**). Stage 4 females were also observed in EE_2 exposures (15%), but this did not differ statistically from controls.

In contrast with that in male fish exposed during the period of sexual differentiation (21-60 dpf), there was a significant effect on/enhancement in gonadal development in females, for both the 100% effluent $(\chi^2)_{(2, N=28)} = 8.1$, $p < 0.05$) and EE_2 $(\chi^2)_{(2, N=26)} = 9.3$, $p < 0.01$) exposures (**Fig. 4Cii**). In females exposed to 100% effluent during the period of gametogenesis to sexual maturation (60-90 dpf) at 90 dpf ovary development was more advanced compared with respective controls $(\chi^2)_{(2, N=23)} = 7.9$, $p < 0.05$), with 23% of females classed as stage 4 versus 0% in respective controls (**Fig. 4Dii**). Stage 4 females were also observed in EE_2 exposures (25%).

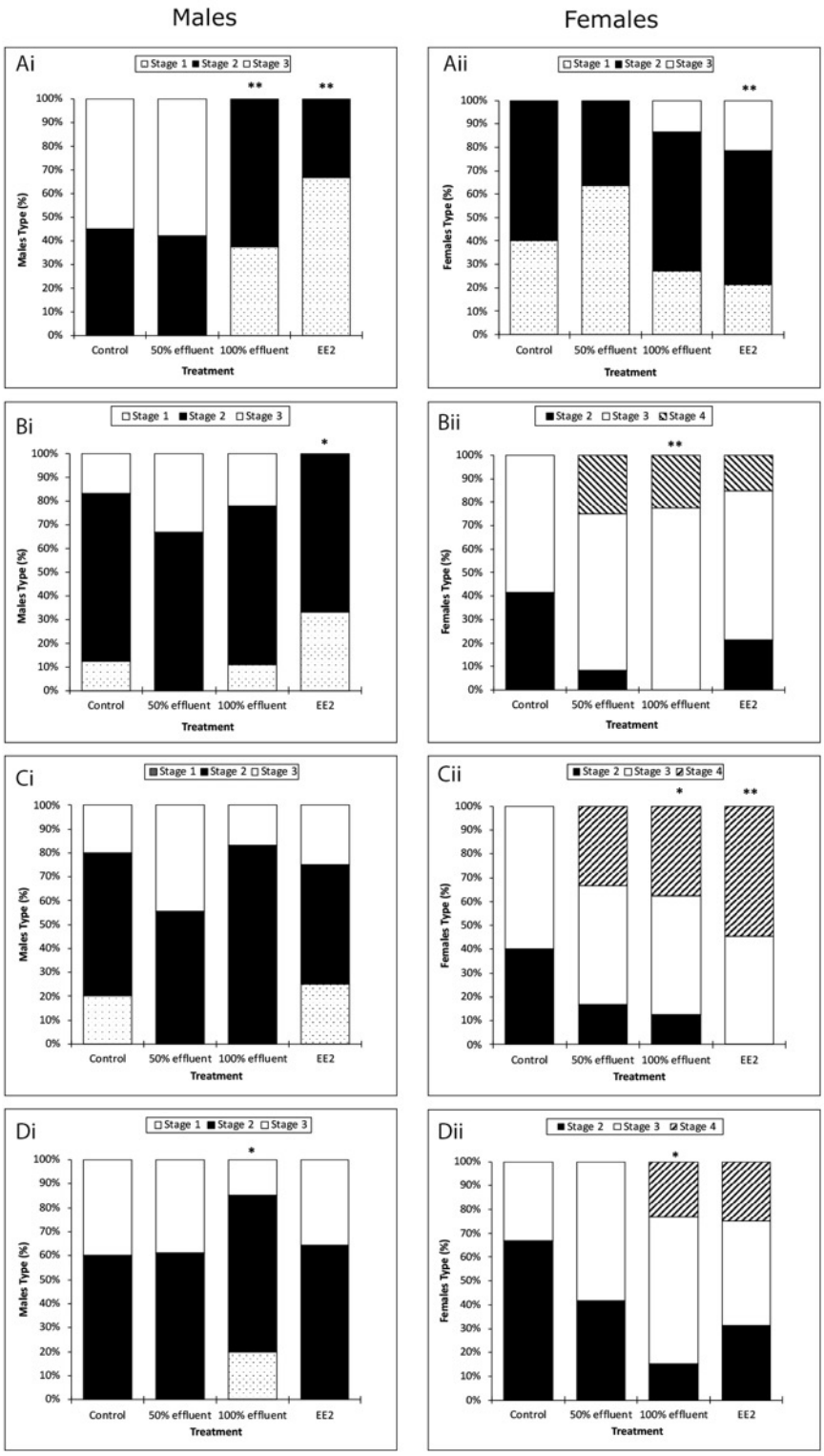

Figure 4. Male and female maturity index in ERE-GFP Casper zebrafish. Data shows transgenic zebrafish at 60dpf or 90dpf in control (unexposed) fish or in fish exposed to EE_2 (10 ng/l) or a graded WwTW effluent concentration during the following developmental periods: (A) 0-90 days (60dpf); (B) 0-90 days (90dpf) (C) 21-60 days (60 dpf) and (D) 60-90 days (90 dpf). Data are reported as mean ± SEM. Asterisks denote statistically significant differences (*p<0.05, **p<0.01) from expected sex/maturity index ratios according to a chi-square contingency table.

3.4.3 GFP expression

0-90-day exposures

In fish exposed to EE_2, in 4 dpf larvae GFP expression was observed in the heart (h), liver (li), somites (sm), forebrain (fb) and in the cardiac muscle (cm) (**Fig. 5Bi-Biii**) with comparable findings at 21dpf (**Fig. 5Ei-Eiii**) and 30dpf (**Fig. 5Hi-Hiii).** At 30 dpf, GFP expression was also seen in the skeletal bone (sc) (**Fig. 5Hiv)**. No specific GFP expression was detected in the forebrain at this time-point. Responses in GFP expression differed for the different developmental time-points in fish exposed to full strength effluent. At 4 dpf, GFP expression was clearly seen in the heart (h) and otic vesicle (ov) only (**Fig. 5Ci-Cii**). In 21 dpf larvae, similar results were recorded, with additional expression of GFP occurring in the somites (**Fig. 5Fi-Fiii**). At 30 dpf, GFP expression was observed in the somites only (**Fig. 5Iii**). In control fish a weak basal GFP expression was observed in in the otic vesicle at both 4 dpf and 21 dpf, and in the heart and in the liver of 30dpf fish.

21-60-day and 60-90-day exposures

In fish exposed to EE_2, for the periods between 21-60-day and 60-90-day comparable findings with that of 0-90 dpf exposed fish were found at 30 dpf. No specific GFP expression was detected in both 50% or 100% effluent exposures at this time point. In those fish over 30 dpf, accurate quantification of GFP expression via fluorescent microscopy was not possible due to skin thickness.

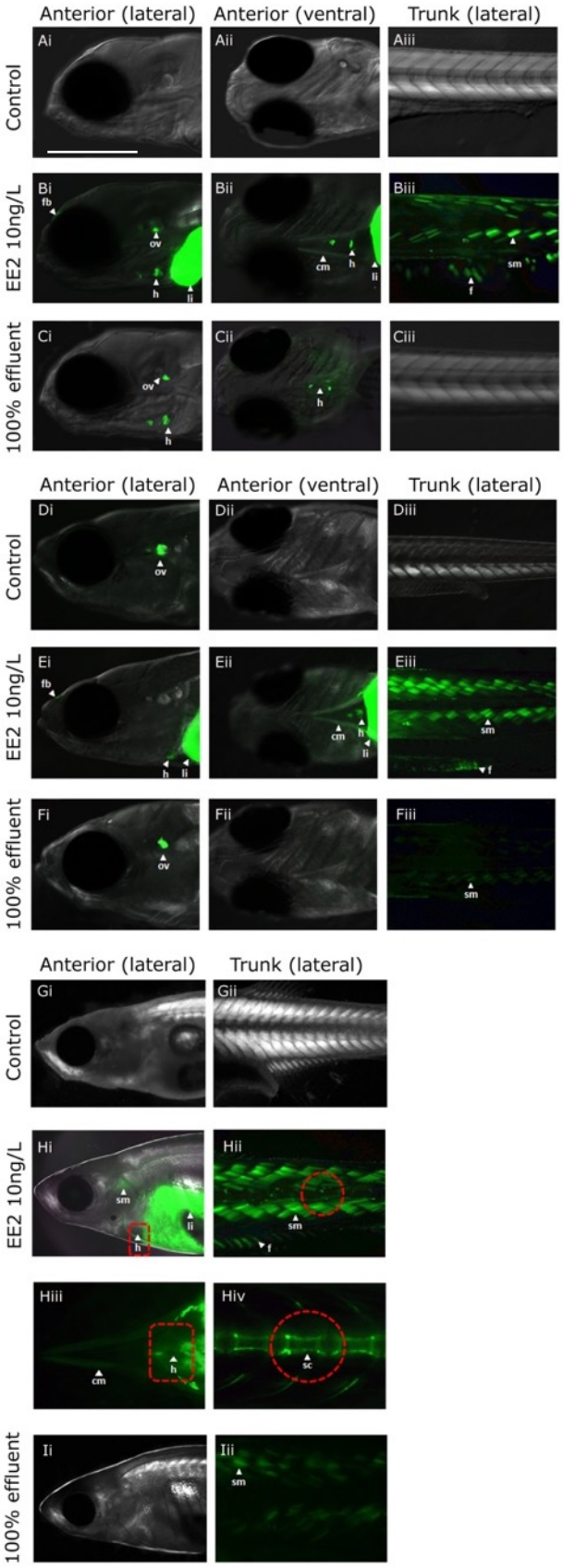

Figure 5. Expression of GFP in different tissue types during different developmental periods in ERE-GFP Casper zebrafish exposed to a wastewater effluent from 0-90 days post fertilisation Transgenic zebrafish at 4dpf in (unexposed) (**A**), EE_2 (10 ng/L, positive control) (**B**), and wastewater effluent (100%) exposed groups (**C**); at 21dpf in (unexposed) (**D**), EE_2 (10 ng/L, positive control) (**E**), and wastewater effluent (100%) exposed groups (**F**); at 30dpf in (unexposed) (**G**), EE_2 (10 ng/L, positive control) (**H**), and wastewater effluent (100%) exposed groups (**I**). GFP expression is shown for regions in the head (lateral – i and ventral – ii views) and trunk (lateral view - iii). GFP induction was observed in the heart (h), liver (li), forebrain (fb), otic vesicle (ov), cranial muscles (cm), somite muscles (sm) and skeletal bone (sc) in the positive control larvae (**B,E,H**), but the wastewater effluent induced different tissue patterns of GFP expression (**C, F, I**). Bar = 200µm.

3.4.4 VTG/GFP mRNA expression

GFP/VTG mRNA induction was measured using whole body ERE-TG Casper zebrafish at 21 dpf and 30 dpf and in the liver in 60 dpf and 90 dpf zebrafish.

0-90-day exposures

In the 0-90 dpf exposure study in fish exposed to EE_2, *gfp* mRNA levels were higher at all time points compared with controls (21 hpf,13.6-fold induction, *p*=<0.05; 35 hpf, 14.3-fold induction, *p*=<0.01; 60 dpf, 14.8-fold induction, *p*=<0.01; 90 hpf,17-fold induction, *p*=<0.001; respectively **Fig. 6Ai**). Levels of *gfp* mRNA in 100% effluent exposures were significantly higher at 60 dpf and 90 dpf compared with their respective controls (3.6-fold, *p*=<0.05 and 2.9-fold, *p*=<0.05, respectively, **Fig. 6Ai**). In addition, no *gfp* mRNA induction was seen at any time point in 50% effluent exposure groups (**Fig. 6Ai**).

Levels of *vtg* mRNA in fish exposed to EE_2 were 15.4-fold higher at 21 dpf (*p*=<0.01), 23.9-fold higher at 30 dpf (*p*=<0.001) 38.2-fold higher at 60 dpf (*p*=<0.001), and 93.2-fold higher at 90 dpf (*p*=<0.001), than in their respective controls (**Fig. 6Aii**). Levels of *vtg* mRNA in 100% effluent exposures were significantly higher at 60 dpf and 90 dpf compared with their respective controls (10.8-fold, *p*=<0.05 and 17.3-fold, *p*=<0.05, respectively, **Fig. 6Aii**). There were no effects for the 50% effluent exposure on levels of *vtg* mRNA induction at any of the measured time points 21, 30 and 60 dpf.

21-60 day exposures

For exposures carried out during the period of sexual differentiation (21-60 dpf), *gfp* mRNA levels were elevated above controls in the EE_2 treatment at 30 dpf and 60 dpf (8.2-fold induction, *p*=<0.01, and 4.5-fold induction *p*=<0.05, respectively **Fig. 6Bi**). For *vtg* mRNA, levels in EE_2 treatments were 22.1-fold higher and 11.9-fold higher at 30 dpf and 60 dpf compared with controls (*p*=<0.01 and *p*=<0.01, respectively). In the 100% effluent treatment levels of *vtg* mRNA were 3.4-fold and 6.4-fold higher compared with their respective controls *p*=<0.05 and *p*=<0.01, respectively). No observable effects on *vtg* mRNA were seen in 50% effluent exposure groups (**Fig. 6Bii**). Levels of *vtg*

mRNA in 30 dpf fish exposed to EE_2 were significantly different compared to 60 dpf fish in the same treatment (p=<0.01, **Fig. 6Cii**).

60-90-day exposures

For exposures carried out to EE_2 during 60-90 dpf, levels of gfp mRNA were higher in the liver when compared with untreated controls (21-fold induction, p=<0.05, **Fig. 6Ci**). There were no differences in *gfp* mRNA for any of the other treatments. *vtg* mRNA in the liver for the exposure to EE_2 was higher compared with controls (89.3-fold, p=<0.001, **Fig. 6Cii**) and also in the liver for the 100% effluent exposure (8.4-fold induction, p=<0.05), with no significant induction observed in 50% effluent exposures.

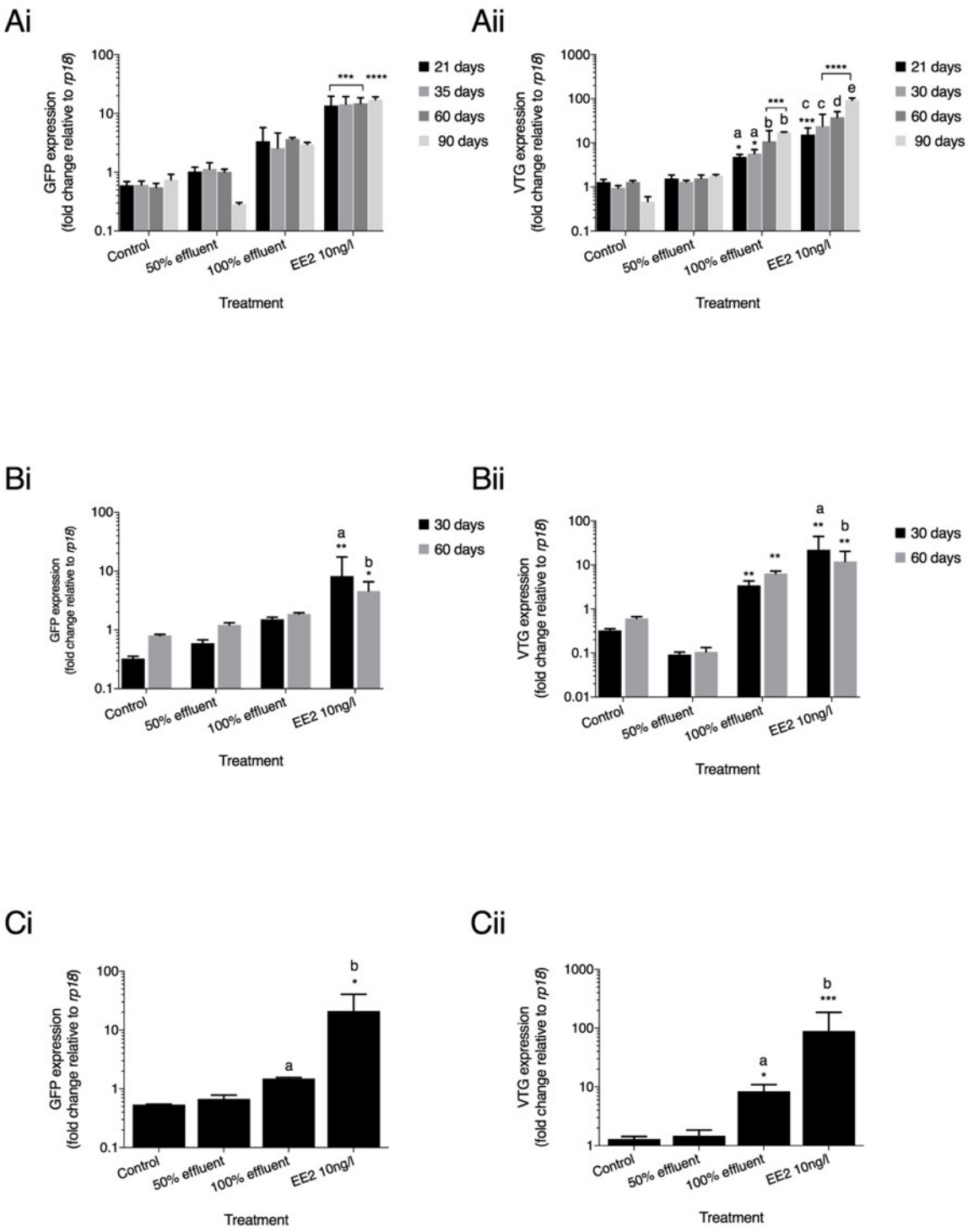

Figure 6. GFP and VTG mRNA expression in ERE-TG Casper zebrafish. Data shows fold change compared with controls following exposure to EE_2 (10 ng/l) or a WwTW effluent (50% or 100%) during the following developmental periods: (A) 0-90 days, (B) 21-60 days, and (C) 60-90 days. Relative mRNA expression was determined as the ratio or target gene mRNA/rpl8 mRNA. Asterisks denote a significant difference (*p<0.05, **p<0.01) compared with control. Within groups, different letters denote a significant difference (p<0.05).

In terms of threshold responses for the concentrations of steroidal oestrogens measured in each of the wastewater effluents, the lowest-observed-effect concentration (LOEC) for *vtg* induction was 19.35 ng/L^{-1} EEQs. For *gfp* expression, the only significant induction, compared with dilution water controls, was for a LOEC of 42.64 ng/L^{-1} EEQs.

A comparison of the induction levels of *gfp* mRNA against *vtg* mRNA in our transgenic zebrafish, showed that the magnitude for responses in *vtg* induction was higher compared to *gfp* for all treatments (**Fig. 7**). Comparison of the data as a whole revealed a positive correlation between the levels of *gfp* expression and the levels of *vtg* expression with differing degrees of strength dependent on the developmental stage measured. For the continuous exposure period of 0-90 days, a weak correlation was observed for both EE_2 (**Fig. 7Ai**) and effluent treatments (**Fig. 7Aii**) [r^2=0.2 and 0.04 respectively]). In fish exposed to EE_2 during the period of sexual differentiation (21-60 dpf), a strong correlation was observed (**Fig. 7Bi**, [r^2=0.8, p=<0.001]), but was much weaker in effluent treatments (**Fig. 7Bii** [r^2=0.2]). For exposures during gametogenesis (60-90 dpf), a strong correlation was again observed in the EE_2 treatment (**Fig. 7Ci**, [r^2=0.7, p=<0.001]), but was more moderate in effluent treatments (**Fig. 7Cii**, [r^2=0.5]).

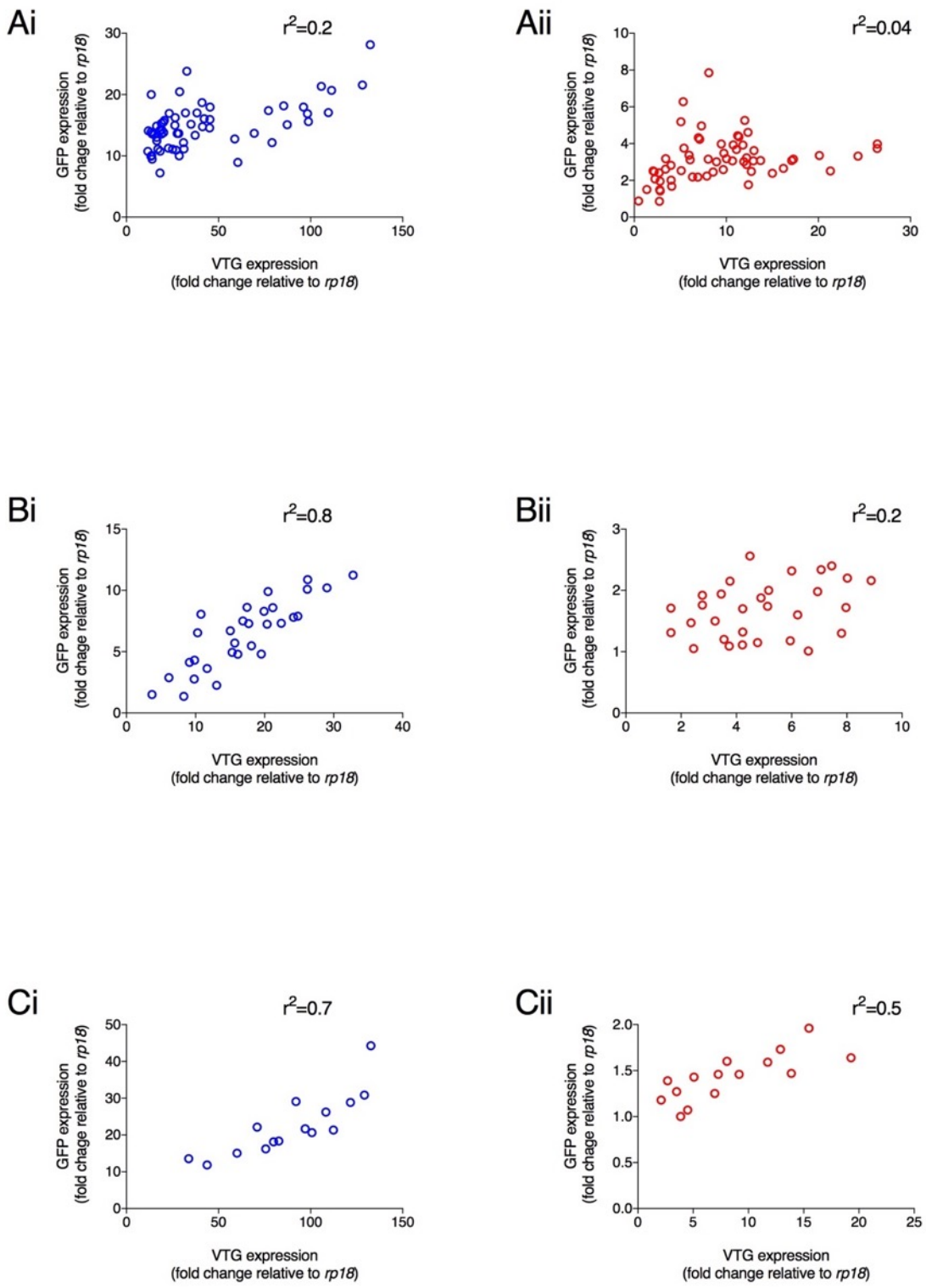

Figure 7. Correlation between GFP and VTG mRNA expression in ERE-GFP Casper. Transgenic zebrafish were exposed to (i) EE_2 (10ng/L) or (ii) a graded effluent (50% and 100%) during the following developmental periods: (A) 0-90 days, (B) 21-60 days, and (C) 60-90 day. Data are reported as mean ± SEM and expressed as fold-change compared with the control. Relative mRNA expression was determined as the ratio or target gene mRNA/rpl8 mRNA.

Full details of the measured levels of *gfp* mRNA against *vtg* mRNA induction, in whole embryo homogenates (21 and 35 dpf) and in the liver of adult zebrafish (60 and 90 dpf), for the different time points measured during the 0-90 day exposure period can be found in the supplementary information (**SI 6**).

Full details of the analysis of E_2 EQs against the induction of *gfp* and *vtg*, are provided in the Supplementary Information (**SI 7**).

3.5 Discussion

EDCs found in the downstream vicinity of WwTWs are associated with reproductive health impacts on fish and some of the key causative chemicals are steroidal oestrogens present in wastewater effluent discharges. The impact of these chemicals on wild fish populations however is still uncertain. In this study, a transgenic zebrafish model -ERE-GFP Casper zebrafish was applied to assess the oestrogenic potency of a WwTW effluent and identify outcomes for both a chronic exposure (0-90 dpf) and acute exposure scenarios incorporating the key developmental stages of sexual differentiation (21-60 pdf) and gametogenesis i.e. sexual maturation (60-90 dpf). Both sex and developmental stage specific effects on somatic growth and sexual differentiation following exposure to 100% effluent were observed, as well as skewed sex ratios in favour of females in all 100% effluent exposure scenarios.

Differing patterns of GFP expression, in terms of target tissues, were observed in zebrafish chronically exposed to 100% effluent. Variations in *gfp*/*vtg* induction were also found in 100% effluent exposures and these were reflective of the measured levels of steroidal oestrogens in the effluent throughout the study periods. Furthermore, when compared with the measurement of *vtg* mRNA, *gfp* mRNA induction was found to be similarly responsive to the wastewater effluent, albeit for *gfp* mRNA there was a lower magnitude of response.

3.5.1 Oestrogenic content of the effluent

In this study effluent mean concentrations of the steroidal oestrogens, E_1, E_2 and EE_2, during chronic exposures (0-90 dpf) were fairly low (7±0 ng/L, 3±0 ng/L and 0.5±0.04 ng/L, respectively) comparable with other weakly estrogenic effluents discharging into UK rivers (Thorpe *et al.,* 2006, 2008). Individually, these levels of steroidal oestrogens are not likely to necessarily induce adverse health effects outcomes, although previous studies have reported induction of VTG and even intersex in some fish species exposed to similar concentrations (Caldwell *et al.,* 2012; Green *et al.,* 2015; Wang *et al.,* 2013; Zhao *et al.,* 2014). Steroidal oestrogens in combination however can be additive in their feminising effects (Silva *et al.,* 2002; Brian *et al.,* 2005). Therefore, a toxic equivalency method was applied with all three steroids combined to produce an EEQs (ng/L), and this gave yield concentrations ranging from 19.35 ng/L to 42.64 ng/L

for the wastewater effluent. These EEQs are reported to be high enough to elicit significant effects on development and sexual differentiation in fish (Bahamonde *et al.,* 2015a; Lange *et al.,* 2011; Tetreault *et al.,* 2011, 2012).

The oestrogenic content of the effluent varied (up to a 2-fold increase) over the 4-month study period with lower concentrations of steroidal oestrogens recorded during the summer months (i.e. June to August) compared with September. These seasonal differences may be due to various factors including differences in temperature and rain fall affecting the effluent dilution within the WwTW. Here, elevated temperatures during the mid-summer period are likely to have improved rates of steroid biodegradation within the WwTW, as suggested in previous studies (Jin *et al.,* 2008; Martinovic *et al.,* 2008; Rho and Chu, 2010). Equally, there were higher levels of rainfall during the months of June to August compared with September and thus a high likelihood for a greater dilution of the effluent and thus EEQs in the works during these times**.** Other factors such as the amount of organic matter (quantified by biological oxygen demand [BOD] and total suspended solids [TSS]), can affect responses in fish exposed to these complex discharges as binding to organic matter affects the bioavailability for uptake into fish, and these were seen to differ, with a lower BOD recorded in the wastewater effluent during September compared to the summer months (June to August).

Overall, analysis of the environmental oestrogens in the wastewater effluent studied emphasises the considerable temporal variability and is concordant with other reports in the literature (Fuzzen *et al.,* 2016; Hemming *et al.,* 2004). Hence, there is a need for multiple measures of extended periods of time to allow for accurate evaluations on the oestrogenic content in WwTW effluents and the potential for effects in wildlife living exposed in receiving rivers

3.5.2 Biological responses in the ERE-GFP zebrafish model

3.5.2.1 Survival and hatching success

For EE_2 no significant effects on survivorship in juvenile or adult zebrafish were found for any of the exposures scenarios and this is concordant with other studies in the literature for this/similar EE_2 exposure concentrations (Versonnen

and Janssen, 2004; Schäfers *et al.,* 2007; Jackson and Klerks, 2019). Conversely, a pronounced mortality for exposure to EE_2 was observed during early development to 48hpf – a period that includes late gastrulation and early organisgenesis, that may indicate a higher sensitivity to oestrogen exposure during this life period and supporting a previous observation in wildtype zebrafish (Soares *et al.,* 2009). Identification of the physiological mechanisms behind this reduced survival was beyond the scope of this study but some associations between the observed effect and EE_2 exposure may be inferred, based on previous findings in the literature. For example, increased mortality may be due to an inability to metabolise oestrogen-induced proteins, such as VTG, which was found to be significantly elevated in zebrafish at all stages of development in this study. This can result in an accumulation of VTG in the kidneys and subsequent renal failure and loss of homeostasis and has previously been linked to exposure to steroidal oestrogens such as EE_2 and E_2 albeit at higher concentrations (Lange *et al.,* 2001; Magliulo *et al.,* 2002; Schäfers *et al* 2007; Thorpe *et al.* 2007). This is one example of an AOP mediated by the oestrogen pathway i.e. ER agonism leading to reduced survival due to renal failure

Increased mortality during this developmental stage may also have been facilitated by an increased chemical uptake of EE_2 due to the onset of 'chorion softening' caused by proteolytic activities found in the pre-hatching stage in zebrafish (Kim *et al.,* 2006). This would increase the permeability in the chorion, as recently illustrated by Kais *et al.,* (2013), who found a time-dependent increase in chorion permeability at 48hpf in unexposed zebrafish embryos, in contrast to that seen at 24 hpf.

Exposure to 10 ng EE_2/L also resulted in reduced hatching success and is in concordance with other studies in the literature (Nash *et al.,* 2004; Versonnen and Janssen, 2004). Again, the precise mechanisms for this effect are unknown, but could be a result of disruptions in the regulation of the hatching gene tetraspanin cd63 (Trikic *et al.,* 2011) by EE_2.

Similarily, for wastewater effluent exposures, mortality rates were not significantly affected in juvenile or adult zebrafish for any of the exposure

periods as found in other WwTW effluent studies (Sowers *et al.,* 2009; Thorpe *et al.,* 2008; Griffin and Harrahy, 2014; McCallum *et al.,* 2017). In comparison, reduced survival was observed in early life stage fish (48-96 hpf) and this again could be due to VTG accumulation in the kidneys (and subsequent renal failure) following exposure to environmental oestrogens commonly found in wastewater effluent such as EE_2, NP and E_2 (Folmar *et al.,* 2001b; Lange *et al.,* 2001; Magliulo *et al.,* 2002). In this study, the individual levels of the steroidal oestrogens present in the wastewater effluent are unlikely to have caused higher levels of VTG induction *per se*, however, in combination their effect can be additive (Silva *et al.,* 2002; Brian *et al.,* 2005), therefore producing an EEQ high enough to account for the observed response.

It is important also to consider that the wastewater effluent used in this study would contain a mixture of many different chemicals, including organochlorine pesticides and pharmaceuticals, any of which may have contributed towards the observed toxic effect in the zebrafish embryos. For example, a study by Li and Lin (2015), found a mixture of 19 commonly used pharmaceuticals caused acute toxicity to European carp (*Cyprinus carpio*) after 96 hpf. Additionally, other physiochemical parameters related to the effluent, in this instance higher levels of suspended solids during the exposure period, may also have contributed to the overall toxic effect via depletion of dissolved oxygen levels.

As with exposures to EE_2, a reduced hatching success was observed in full strength (100%) effluent exposures as previously reported in the literature (Brazzola *et al.,* 2014; Smolders *et al.,* 2002). Again, the precise mechanisms for this effect are unknown, but could be a possible inhibition/decrease in proteolytic enzymes (i.e. choriolysin) which are required for successful hatching (Kim *et al.,* 2006; Sano *et al.,* 2008). In a recent study by De la Paz *et al.,* (2017), exposure to the fungicide triazole, a selective oestrogen receptor modulator (SERM), was found to lead to a strong inhibition of hatching in zebrafish embryos. This was correlated with a reduction in the secretion of hatching gland cells, which have previously been shown to express all three ER subtypes during early zebrafish life stages (Chandrasekar *et al.,* 2010; Hao *et al.,* 2013).

3.5.2.2 Effects on somatic growth

The presence of oestrogens during specific developmental stages (i.e. sexual differentiation), are known to be crucial for both normal gonadal development and somatic growth in fish (Leet *et al.,* 2011). Here, it was shown that chronic exposure to EE_2 (0-90 dpf) resulted in a reduced somatic growth in both males and females, supporting previous findings for chronic exposures in fish (Schäfers *et al.,* 2007; Shved *et al.,* 2007, 2008; Xu et al., 2008). It was further shown that even relatively short-term exposures during the period of sexual differentiation (21-60 dpf) and gametogenesis/sexual maturation (60-90 dpf) similarly led to reduced growth in both sexes (as per Silva *et al.,* 2012). In the literature, EE_2 has also been reported to have growth-promoting capability in fish, albeit measured concentrations used in these studies were lower, ranging from 0.1 to 4ng EE_2 L^{-1} (Chen *et al.,* 2017; Luzio *et al.,* 2015; Luzio *et al.,* 2016; Orn *et al.,* 2003). Aligning these inconsistencies between studies is difficult but is suggestive of a concentration dependent effect on fish growth that is typical of a non-monotonic concentration response behaviour, often reported for EE_2, and appears to be common for EDCs in general (see review – Vandenburg *et al.,* 2012). For example, in a study by Larsen *et al.,* (2008), comparisons of average body weight in zebrafish found that males exposed to 0.5 ng EE_2 L^{-1}, had a lower body weight in comparison to males exposed to 5 ng EE_2 L^{-1}, who in turn displayed higher body weights compared to unexposed controls. Additionally, differing results between studies may be a result of reduced exposure times, and/or higher water temperatures. For example, Luzio *et al.,* 2016, found that zebrafish exposed from 0-60 dpf to 4.ng EE_2 L^{-1} at 33 ^{o}C were significantly larger than those exposed to the same concentration at 28 ^{o}C.

Both chronic (0-90 dpf) and short-term (21-60 dpf) exposures to the 100% WwTW effluent resulted in enhanced growth in both male and female zebrafish, corroborating previous findings for other effluent exposures (Liney *et al.,* 2005, 2006) and may have been due to growth promoting chemicals (i.e. pharmaceuticals) found in the wastewater effluent. Whilst this may seem to appear as beneficial, there is evidence that rapid growth in fish can affect locomotor performance in a number of taxa, as a result of effects on muscle cellularity and development (Lee *et al.,* 2010). This is important as many fish behaviours such as anti-predator avoidance or breeding performance via sexual

displays is strongly related to swimming performance (Billerbeck *et al.,* 2001; Royle *et al.,* 2006).

Additional nutritional sources present in the effluent (in this instance consisting of a biofilm) may also have contributed to the observed enhancement in growth in the zebrafish model. Biofilms formed during wastewater treatment can include microorganisms such as phytoplankton and zooplankton. Equally, there are other studies that have reported a decrease in somatic growth (Smolders *et al.,* 2002), and this was seen in exposures to 100% effluent during the period of gametogenesis/sexual maturation (60-90 dpf). Due to the sheer number and variation of organic and inorganic chemicals found in any one wastewater effluent it is difficult to elucidate with any real confidence whether the disparate growth effects seen in the ERE-GFP Casper zebrafish were a result of steroidal oestrogens alone and is highly improbable. Mechanistically, growing evidence suggests that effects on somatic growth rates (and on reproduction) for environmental exposure to oestrogens in fish operate through the growth hormone/insulin-like growth factor 1 (GH/IGF) pathway (Filby *et al.,* 2006; Goetz *et al.,* 2009; Segner *et al.,* 2006; Shved *et al.,* 2007, 2008). In a recent study by Hanson *et al.,* 2017, depressed growth in adult rainbow trout exposed to E_2, β-sitosterol (βS) and NP (levels) was suggested as occurring via a reduction in GH sensitivity in terms of reduced growth hormone receptor (GHR) synthesis and surface GHR expression, caused by inhibition of gene expression elements of the GH/IGF system. Similar results were also previously reported by the same author (Hanson *et al.,* 2014) and in a study by Davis *et al.,* 2008.

It is therefore feasible then that somatic growth in the zebrafish may have been affected by oestrogenic chemicals found in the effluent at the time of exposure, leading to a decrease in body size. This is further supported by temporal differences in the chemical makeup of the effluent, which saw a 2-fold increase in oestrogenic activity during this exposure period (i.e. 60-90 dpf). Alternatively, these results may simply be attributable to general toxicity effects caused by other chemicals present in the effluent. Exposure of waterborne trace metals such as copper and cobalt for example, have been shown to cause significantly lower weight and fork length in some fish species (Authman *et al.,* 2015; Javed, 2013) and are commonly found in wastewater effluents.

In this study zebrafish showed the sexual dimorphic growth patterns and body shape well established for this species (Lawrence *et al.,* 2008; Santos *et al.,* 2008) and females were larger than males for all exposure groups. However, growth effects were more pronounced for the treatment groups in males than in females, with significant smaller sized fish seen for exposures to EE_2 at all time points and during 60-90-day 100% effluent exposures. This serves to highlight differences in effects that occur between the sexes for somatic tissues, which in turn may also indirectly affect rates of sexual development that can show a size dependency. For example, in this study, all EE_2 exposures resulted in a reduced growth in male zebrafish and were also associated with a delay in gonadal maturation. Growth and sexual maturation have been shown to correlate closely to each other in zebrafish (Baumann *et al.,* 2008; Chen and Ge, 2013**;** Schäfers *et al.,* 2007) as well as in other gonochoristic fish species, such as the fathead minnow (Länge *et al.,* 2001) and are thus likely to sway the expression of genes related to the hormonal system via some form of mechanistic relationship between the ER pathway and IGF-1 gene expression (Shved *et al.,* 2008). Body size in fish is a feature that has a positive relationship with fitness components such as survival and fecundity (Kingsolver and Pfennig, 2004) and thus, a reduced body mass could potentially reduce life time fitness, (i.e. the total number of offspring produced during an individual's lifetime), with subsequent implications for population level effects (Johnson and Hixon, 2011).

3.5.3 Effects on gonadal development

Chronic exposure to both EE_2 and 100% effluent from early fertilisation to sexual maturation (0-90 dpf) was shown to delay the maturation of testis in males, and this was in contrast with females where the opposite effect was observed (i.e. an accelerated gonadal development), and is comparable with other studies in the literature (Fenske *et al.,* 2005; Barber *et al.,* 2007; Baumann *et al.,* 2013, 2014a; Luzio *et al.,* 2015, 2016). These findings were consistent for all developmental exposure periods in both these groups and demonstrates how oestrogens can elicit distinct effects dependent on sex. Information on the mechanisms by which exogenous oestrogens impact gonad maturation is limited. Gonadal sex differentiation in teleost fish is believed to be highly dependent on endogenous levels of oestrogens (and androgens) in determining phenotypic sex, in such a way that excess E_2 induces feminine differentiation,

while excess 11-ketotestosterone (11KT) induces masculine differentiation (Fenske and Segner 2004; Guerrero-Estévez and Moreno-Mendoza 2010). Exposure to exogenous oestrogens (such as at those levels found in this study) can disrupt this hormonal balance, by interfering with steroidogenic enzyme expression or activity (Deng *et al.,* 2010; Fenske and Segner, 2004; Muth-Köhne *et al.,* 2016; Suzawa and Ingraham, 2008), or through agonistic or antagonistic binding to ER receptors resulting in the disruption of normal gonadal differentiation and maturation. Oestrogens such as E_2 also stimulate *vtg* production in teleost fish, primarily through the activation of *esr1* and down regulation of the GH-ICF1 pathway and therefore energy normally used for testicular differentiation in the ERE-GFP Casper zebrafish may instead have been utilised for *vtg* synthesis, as evidenced by the increased levels of *vtg* mRNA found in both EE_2 and 100% effluent exposed groups, thus delaying the maturation of the testis in males. This has also been evidenced in a study by Davis *et al.,* (2008) who proposed that induction of *vtg* by E_2 in male tilapia (*Oreochromis mossambicus*), shifted energy from gonadal growth towards vitellogenesis in the liver.

3.5.3 Sex ratios

Sex ratio is a key endpoint when evaluating the feminizing effects (or masculinization) in fish exposed to EDCs (OECD, 2010). In the current study, a clear bias towards females was found for all developmental exposure periods (compared to untreated controls) in both EE_2 and 100% effluent exposed groups. This is to be expected given the level(s) of both EE_2 and of the steroidal oestrogens present in the WwTw effluent, which have previously been reported as being sufficient to skew sex ratios in favour of females (Baumann *et al.,* 2013, 2014a; Chen *et al.,* 2015a; Naderi *et al.,* 2014; Xu *et al.,* 2008). The observed developmental bias towards females in 100% effluent exposures may also have been influenced by the additional nutritional sources present in the effluent, allowing for more energy to be assigned for gonadal development (Barber *et al.,* 2007; Lawrence *et al.,* 2008). In zebrafish, an accelerated growth rate, during the critical period of gonadal differentiation, is thought to be either a trigger of or a key influence in successful ovarian differentiation (Baroiller *et al.,* 2009). Indeed, the idea that growth performs a primordial role in sex determination in fish is not a new concept (Kraak and de Looze, 1993), with

numerous reports of higher proportions of females amongst the largest fish found within wild populations (Saillant *et al.,* 2003a; Vandeputte *et al.,* 2007).

Determining the exact mechanisms underlying the observed effects on sex ratios found in this study are difficult to elucidate but are again likely linked to disruptions in hormone levels by exogenous levels of oestrogens, resulting in a possible arrestment of development in males as shown in previous studies (Baumann *et al.,* 2013; 2014; Van der Kraak and Lister, 2011). However, genetic determination of the gender of an individual zebrafish would be needed to confirm this and is currently not possible due to the lack of a sex marker for this species, which could help distinguish between phenotypic and genetic sex. Feminization in males following exposure to exogenous oestrogens can also occur as the result of full sex reversal. For example, Lange *et al.,* (2011), found that chronic exposure of roach (*Rutilus rutilius*) to a full strength wastewater effluent led to sex reversal in almost all males within the population, whilst a 28 day investigation by Vadja *et al.,* (2011), with adult fathead minnows (*Pimephales promelas*), reported a rapid sex reversal of males exposed to a WwTW effluent, albeit the EEQs for this effluent were moderately higher (averaging 50 ng EEQs/L) in comparison to those found in this study. Given the absence of any intersex fish in any of the exposure scenarios in this study it is highly likely that the observed effects on sex ratios in favour of females were a result of sex reversal and not an arrestment of development in male zebrafish.

Several studies have shown that sex reversal in zebrafish is highly dependent of the timing and duration of exposure. For instance, exposure to EE_2 at levels similar to those in this study during the period of gonad differentiation (20-60 dpf) can result in either complete feminization or a significant change of the sex ratio towards females (Andersen *et al.,* 2003; Brion *et al.,* 2004) and this suggests that the critical period at which EE_2 has a feminizing effect on the differentiation of the gonads in zebrafish is during this development stage. In most teleost fish the gonads maintain bi-potentiality, even after gonadal differentiation (Devlin and Nagahama, 2002), and thus there is a potential of exogenous oestrogens to cause sex reversal in later life stages or in adults. In this study, a shift of sex ratios towards females in 90 dpf zebrafish was observed, in both chronic (0-90 dpf) and acute (60-90 dpf) exposures for EE_2,

although these were less pronounced in the latter (6:7 and 1:11 respectively compared to the control population where there was a 2:1 bias towards males), suggesting a lower sensitivity of the gonads once the process of sexual differentiation has ended.

3.5.4 Intersex

Another signature effect in gonochoristic fish often associated with exposure to steroidal oestrogens is intersex (or the presence of oocytes in the testes). In this study, no incidences of intersex fish were seen at any time-point in EE_2 exposure groups, and this is in concordance with other studies (Andersen *et al.,* 2003; Örn *et al.,* 2003; Fenske *et al.,* 2005; Luzio *et al.,* 2016). Conversely, EE_2 has also been linked to intersex in fish, although this is largely reported for other fish species (Depiereux *et al.,* 2014; Hirakawa *et al.,* 2013) and/or in combination with other chemicals (Luzio *et al.,* 2015a). For example, a study by Orn *at al.,* 2003, showed species differences between medaka and zebrafish exposed to the same concentration of 10 ng EE_2 $/L^{-1}$, with the latter displaying no instances of intersex vs 10% intersex in medaka. Unlike zebrafish, the process of gonadal transformation and sex differentiation in medaka is much more fixed, with all fish possessing an undifferentiated gonad which develops directly into mature ovaries or testes, making them more pliable to the intersex condition. In contrast, sex differentiation in zebrafish is far more complex, with all individuals, irrespective of their genetic background, possessing juvenile ovaries prior to the final differentiation of mature ovaries or testes (Maack and Segner, 2003; Wang *et al.,* 2007). Recent studies have also found that not all male zebrafish go through the juvenile ovary to testis transformation process (Luzio *et al.,* 2015b, 2016b; Wang *et al.,* 2007) further highlighting the complexity of sex differentiation in this species and brings into question the suitability of the zebrafish for studies into the health effects of oestrogenic chemicals in relation to sexual development.

Intersex in fish has also been widely documented in fish following exposure to WwTW effluent (Bahamonde *et al.,* 2015a; Woodling *et al.* 2006, Vajda *et al.* 2008). In the current work, no evidence of intersex fish was observed in either 50% or 100% effluent exposures at any of the measured developmental time-points, in direct contrast to these previous findings, and this may be a reflection

of other chemicals (e.g. anti-oestrogens) negating the effects of exogenous oestrogens found in the effluent. However, the high incidence of sex reversal towards females in the ERE-GFP Casper zebrafish suggests that the levels of exogenous oestrogens in the wastewater effluent were sufficient to completely override endogenous endocrine signals, hence resulting in an absence of intersex fish in this study.

It is worth noting that the effluent exposures in this study were carried out under strictly controlled conditions, which required an acclimation of the effluent to a desired temperature of 28 ^{o}C, hence some degree of chemical degradation would be expected. However, this would have been minimal due to the continuous flow through method employed in this study. In comparison, in all of the aforementioned studies effluent exposures were either carried out in the field with caged fish or were conducted with wild fish collected from sites downstream of WwTWs. Fish would therefore have been subject to variable environmental parameters such as fluctuations in temperature and precipitation, other exposure routes of chemical pollutants, (i.e. agricultural run-off and combined sewer overflow events) and also longer exposure periods (in the case of wild fish collected from the field). Hence, this could account for the discrepancies seen between studies.

Intersex does not generally occur in fish that have completed gonad differentiation (Bahamonde *et al.,* 2013) and this would account for the results seen in this study in the 60-90 dpf exposure period, with both effluent and EE_2 treatments.

3.5.5 GFP expression

In this study, application of the pigment free, ERE-GFP-Casper zebrafish model, allowed for identification of variations in GFP responses in tissue types over time and for different developmental life stages, after exposure to a complex environmental mixture and to an individual oestrogen.

In EE_2 exposed fish, tissue responses were observed in the liver, heart, somites, forebrain, otic vesicle and in the cardiac muscle, at 4 dpf, in concordance with previous findings (Lee *et al.,* 2012; Green *et al.,* 2016; our own unpublished data),

with similar responses at 21 and 30 dpf and this corresponded well with increased levels of *gfp* mRNA induction. However, in 30 dpf exposed fish, additional GFP expression was also present in the skeletal bone (i.e. mineralised tissue). Several studies suggest that environmental oestrogens, such as EE_2, BPA and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), can impact on skeletal development and morphology in fish (Ahi *et al.,* 2016; Pinto *et al.,* 2014; Warner and Jenkins, 2007). In addition, a study by Rawson *et al.,* (2008), found significant changes in hemal spine morphology in the mosquitofish, *Gambusia* spp, living downstream of a WwTW, indicative of some form of oestrogenic effect of the final wastewater effluent on development. Here, the sensitivity of the skeletal tissue to EE_2 suggests that exposure to oestrogenic chemicals could have implications for bone formation and integrity in fish. The results of this study also suggest that juveniles undergoing sexual differentiation may be particularly sensitive to EE_2, with a wider range of tissue targets responding compared with larval stages.

In contrast, chronic exposure to 100% effluent induced GFP expression in the heart, otic vesicle and somites only. This was coupled with differences in tissue patterning at each developmental stage, e.g. at 30 dpf, GFP expression was observed in the somites only. These results may be attributable to the presence of ER antagonists in the effluent and/or other chemicals that inhibit a section of the oestrogen responsive pathway (e.g., the activation of the EREs), thereby altering the oestrogenic potency of the mixture. In addition, known differences in ER ligand affinity between environmental oestrogens may well influence physiological targets and subsequent downstream effects (Cosnefroy *et al.*, 2012).

Interestingly, no GFP induction was observed in 30d pf fish exposed to 100% effluent during the period of sexual differentiation (21-60 dpf). This was most likely due to the shorter duration of the exposure period, in combination with low measured concentrations for the three steroidal oestrogens found in the wastewater at this particular period in the study (ranging from 0.5 -2.0 ng/L) and suggests that a longer exposure time is required to induce GFP expression in weaker oestrogenic effluents. No significant induction of GFP expression was seen at any time point in fish exposed to 50% effluent.

One of the major limitations of the previous transgenic ERE-GFP Zebrafish model was the inability to accurately observe GFP expression within internal tissues post 4 dpf, (i.e. in juvenile and adult zebrafish) using fluorescent microscopy. This was due to skin thickness and pigmentation in normal zebrafish development. Whilst the development of the pigment free ERE-GFP Casper has helped with this problem, it has not completely resolved it, as accurate quantification of GFP expression via fluorescent microscopy was not possible in live fish over 30pdf due to skin thickness. Consequently, and unlike for early life stages, effects analysis could not be carried out in live adult fish, thus imposing restrictions on the possible future utility of the model in later life stages.

Overall, this study has demonstrated that one of the major advantages of oestrogen responsive biosensor transgenic fish is their ability to visually detect tissue-specific effects of environmental oestrogens, through the binding of a chemical and subsequent activation of the ERs. This is a classic example of a MIE and demonstrates the potential of the zebrafish model used here in terms of defining AOPs for this group of chemicals. For example, several potential biomarkers (or KEs) are described in this study that could be used to evidence exposure to an oestrogenic chemical operating through the AOP for oestrogen activation, including increased *vtg* production, impaired development and sex reversal in males.

3.5.6 VTG induction

Incidences of elevated levels of VTG expression in wild fish found at sites downstream from WwTWs are frequently reported in the literature and are highly associated with exposure to environmental oestrogens (Brooks *et al.,* 2006; Kavanagh *et al.,* 2004; Vajda *et al.,* 2008; Woodling *et al.,* 2006). In this study, an increase in *vtg* mRNA expression over time in fish chronically exposed to both 100% effluent and EE_2, was observed suggestive of a gradual accumulation of an environmental oestrogen(s), allowing for the observed response, and/or of a greater sensitivity of *vtg* induction. Equally, studies with zebrafish chronically exposed to EE_2, have demonstrated a reduced VTG responsiveness. For instance, Nash *et al.,* 2004, found that life-long exposure to 5 ng EE_2/L^{-1} led to a reduced vitellogenic response in zebrafish (in second

generation males) indicating a possible acclimation of the fish to the EE_2 exposure.

These findings may also indicate differences in gene-specific (*vtg1*) sensitivities during zebrafish development. Indeed, evidence suggests that the expression of VTG is life stage dependent, with high variation found between the sexes and between tissue type (Mikawa *et al.,* 2006; Scholz *et al.,* 2004; Wang *et al.,* 2005). It is interesting to note that the zebrafish genome contains at least 7 *vtg* genes all of which are expressed in the liver of female fish and are inducible in the liver of males by exogenous oestrogens as described by Wang *et al.,* 2005. This author also found extrahepatic expression of *vtg* genes (predominately *vtg1* and *vtg2*) including for the intestine and ovary in females, and in the testis, muscle and intestine in E_2 treated males and this may account for the induction of *vtg* in early life stage fish (21 dpf) seen in this study. The induction of *vtg* in early life stages, before the onset of sexual differentiation, has also been reported in other studies in the literature (Bogers *et al.,* 2006, Xu *et al.,* 2008), including for embryonic life stages (Jin *et al.,* 2009; Cooper *et al*. unpublished data).

Chronic and short-term exposure to EE_2 also resulted in significant increases in *vtg* mRNA expression in the liver of adult zebrafish, in concurrence with other studies (Caspillo *et al.,* 2014; Jin *et al.,* 2009; Martyniuk *et al.* 2007; Soares *et al.,* 2009) and which has also been described for a variety of fish species including rainbow trout, and medaka (Gunnarsson *et al.,* 2007; Zhang *et al.,* 2008) and this is to be expected given the major role of the liver in vitellogenesis in fish (Hara *et al.,* 2016). A higher induction of *vtg* in later life stages however, may not necessarily indicate a higher sensitivity to estrogenic exposure. For example, a study by Jin *et al.,* (2009) found that early stage zebrafish were more sensitive (lower LOECs for *vtg* induction) to the presence of NP compared to juvenile and adults who were more effective in their response (i.e. higher induction levels).

The present study compares, for the first time, the induction of *vtg* mRNA gene expression and corresponding *gfp* mRNA gene expression levels, by an environmental oestrogen(s) during different developmental stages, using a

transgenic zebrafish model. Both biomarkers detected environmentally relevant concentrations of oestrogen(s) for EE_2 and contained within an oestrogenic effluent. The range of the response for the induction of VTG however was far greater when compared to GFP (as previously reported by this author for early life stage embryo zebrafish) reflecting disparities in the dynamics of synthesis and turnover in mRNA between the two genes.

Interestingly, when levels of *vtg* and *gfp* mRNA induction for both 100% effluent and EE_2 exposures were correlated for the whole of the chronic (90 day) exposure period, there was a weak correlation (r^2 = 0.2 and 0.04 respectively) between the two variables, which was surprising. In contrast, when *vtg* and *gfp* mRNA induction was compared for specific life stages during this exposure period, i.e. at 21-30 day and for 30-60 days the correlations were stronger (r^2 = 0.6 and 0.7 for EE_2 & 0.5 and 0.6 for 100% effluent exposures respectively, SI, Fig. S6). This may be explained by the possibility of a weaker correlation between *vtg* and *gfp* mRNA induction in fish up to 21 dpf, a period prior to sexual differentiation, where the hepatic *vtg* gene may be less responsive to oestrogen stimulation. It is worth emphasising also here that the *gfp* mRNA response occurs across multiple tissues for the different life stages, whereas for *vtg* mRNA induction is exclusive to the liver.

3.7 Conclusion

In summary, the results of this investigation show that the health impacts of environmental oestrogens on zebrafish vary with exposure concentration, duration and across different developmental life stages. The exposures support previous observations that period of gonad differentiation in zebrafish is a sensitive window to the actions of exogenous oestrogens and highlights the importance of testing this life stage when evaluating endocrine disrupting effects. As a single biomarker, these results demonstrate that VTG remains, to date, the most sensitive marker known for exposure to oestrogens. However, the ERE-GFP-Casper zebrafish model offers easy to use experimental model, for observing target tissues for environmental oestrogens, through quantifiable GFP fluorescence in live fish. Furthermore, it offers the potential to facilitate the simultaneous understanding of chemical mechanisms of action and adverse outcome analyses from the molecular to the organismal level, thus proving its

viability as a tractable system which can be useful to the fields of ecotoxicology and environmental risk assessment (ERA).

As aquatic organisms are exposed to complex chemical mixtures, rather than to one pollutant at a time, understanding the toxicological effects, mechanisms, and potential wider health impacts of environmental oestrogens (and consequently how this may influence wild fish populations) is vital for effective future conservation and management of freshwater resources.

3.8 References

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3.11 Supplementary Information

SI 1. Generation of ERE-GFP-Casper (F0) line. TG1 indicates ERE-Gal4ff transgene sequence. TG2 indicates UAS-GFP transgene sequence. Expression of pigmentation genes *roy* (dark) and *nacre* (silver) are also shown. The ERE-GFP model, homozygous for both transgenes, and a Casper strain were initially crossed to give a heterozygous generation. Larvae were then screened for the homozygous ERE/Casper genotype and individuals carrying both the Casper phenotype and GFP expression were raised to sexual maturity. Fish were then screened for homozygous expression of the transgene sequences to produce the founder generation (F0) of the ERE-GFP-Casper line.

SI 2. Diagrammatic representation of the exposure system used for delivery of the effluent from the storage tank to the exposure tanks via the glass mixing vessels. A series of peristaltic pumps were used to deliver the effluent, dilution water and oestrogen positive control to the respective mixing vessels and then from each mixing vessel to the replicate exposure tanks for each graded effluent treatment (50 and 100% effluent) and the dilution water control (DWC) and positive control (EE2). The treatments were randomly distributed within the exposure room but are shown here ordered for ease of presentation.

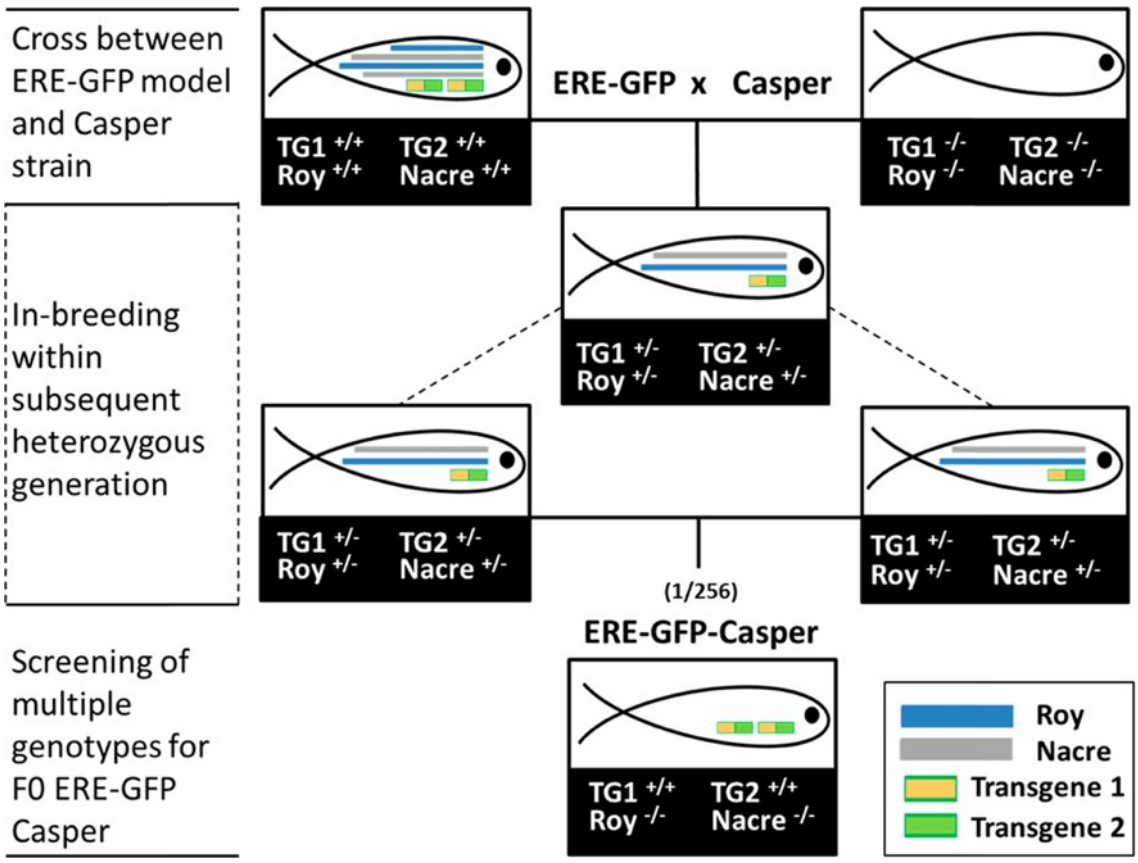
SI 3. Summary of treatment process type, including total hydraulic retention times (HRT) and sludge retention times (SRT), total population equivalent (p.e.) values and types of industrial discharges for the studied treatment works. Data is reported as mean ± SEM.

SI 4 Protocol description of LC-MS used for chemical detection of the steroidal oestrogens in the WwTW effluent.

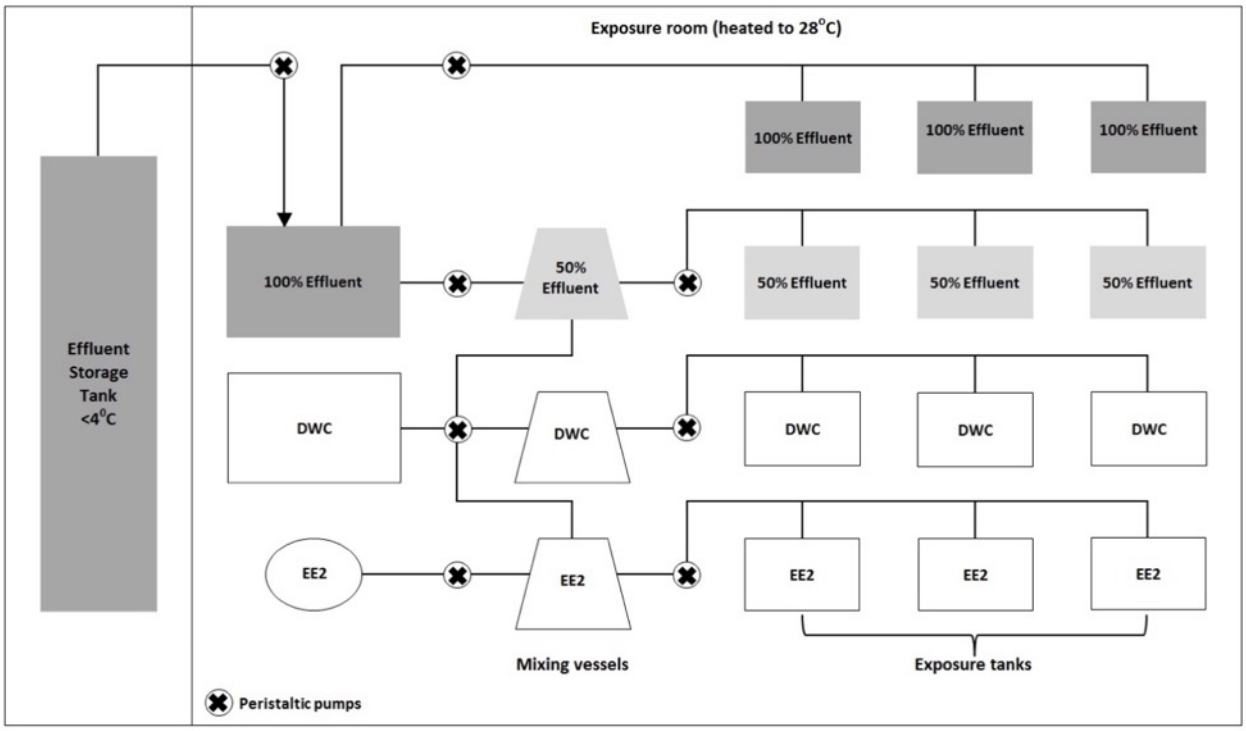
SI 5A/B. Different germ cell types observed during the spermatogenic process (A) and oogenesis (B) in ERE-TG Casper Zebrafish. The maturity staging of gonadal development was assigned based on OECD guidelines by means of a numerical staging system i.e. ovary: stages 0-5, testis: stages 0-4.

SI 6. Correlation between GFP and VTG mRNA expression in ERE-GFP Casper at different time points in a 0 to 90-day chronic exposure. Transgenic zebrafish were exposed to (i) EE_2 (10ng/L) or (ii) a graded effluent (50% and 100%) and sampled at (A) 21dpf, (B) 30dpf, (C), 60dpf, and (D) 90dpf. Data are reported as mean ± SEM and expressed as fold-change compared with the control. Relative mRNA expression was determined as the ratio or target gene mRNA/rpl8 mRNA.

SI 7. Comparison of predicted E2 EQs calculated on the measured concentrations of the three target oestrogenic chemicals in the individual effluents and their relative oestrogenic potencies for the induction of VTG/GFP in zebrafish.


Cross between ERE-GFP model and Casper strain
In-breeding within subsequent heterozygous generation
Screening of multiple genotypes for F0 ERE-GFP Casper
ERE-GFP x Casper
TG1 +/+ Roy +/+
TG2 +/+ Nacre +/+
TG1 -/- Roy -/-
TG2 -/- Nacre -/-
TG1 +/- Roy +/-
TG2 +/- Nacre +/-
TG1 +/- Roy +/-
TG2 +/- Nacre +/-
TG1 +/- Roy +/-
TG2 +/- Nacre +/-
(1/256)
ERE-GFP-Casper
TG1 +/+ Roy -/-
TG2 +/+ Nacre -/-
Roy
Nacre
Transgene 1
Transgene 2

SI 1.


Exposure room (heated to 28°C)
Effluent Storage Tank <4°C
100% Effluent
100% Effluent
100% Effluent
100% Effluent
50% Effluent
50% Effluent
50% Effluent
50% Effluent
DWC
DWC
DWC
DWC
DWC
EE2
EE2
EE2
EE2
EE2
Mixing vessels
Exposure tanks
Peristaltic pumps

SI 2.

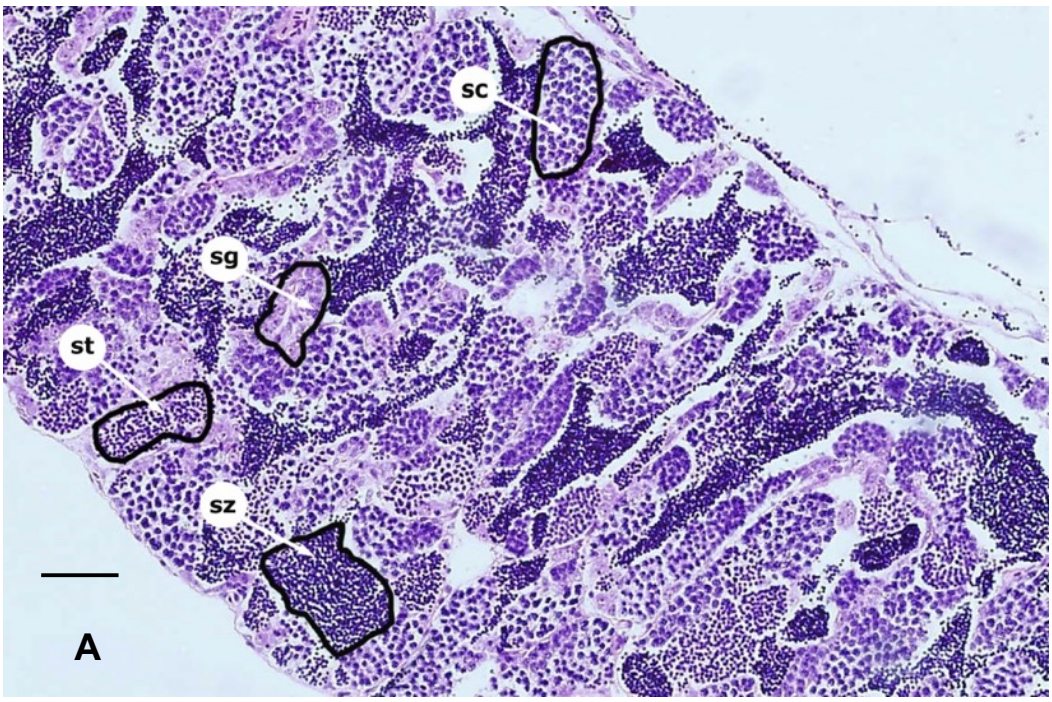
Exeter WwTW	
Total population equivalent	141698
Industrial population equivalent	4713
Proportion of industrial wastewater (%)	3.30%
Secondary treatment	Activated sludge
Tertiary treatment	Nitrifying BAFF
	UV Disinfection
Total HRT (hrs)	6 AS/12 BF
Total SRT (days)	6
Number of permitted industrial/trade discharges	49
Types of industrial/trade waste	Car washes
	Plant wash down
	Laundrettes
	Rendering plant
	Electroplater
	University of Exeter
	Cattle market
	Gas holders
	Swimming pools
	Food processing

BAFF = Biologically aerated flooded filter
AS = Activated sludge
BF = Biological filters
HRT = Hydraulic retention time
SRT = Sludge retention time

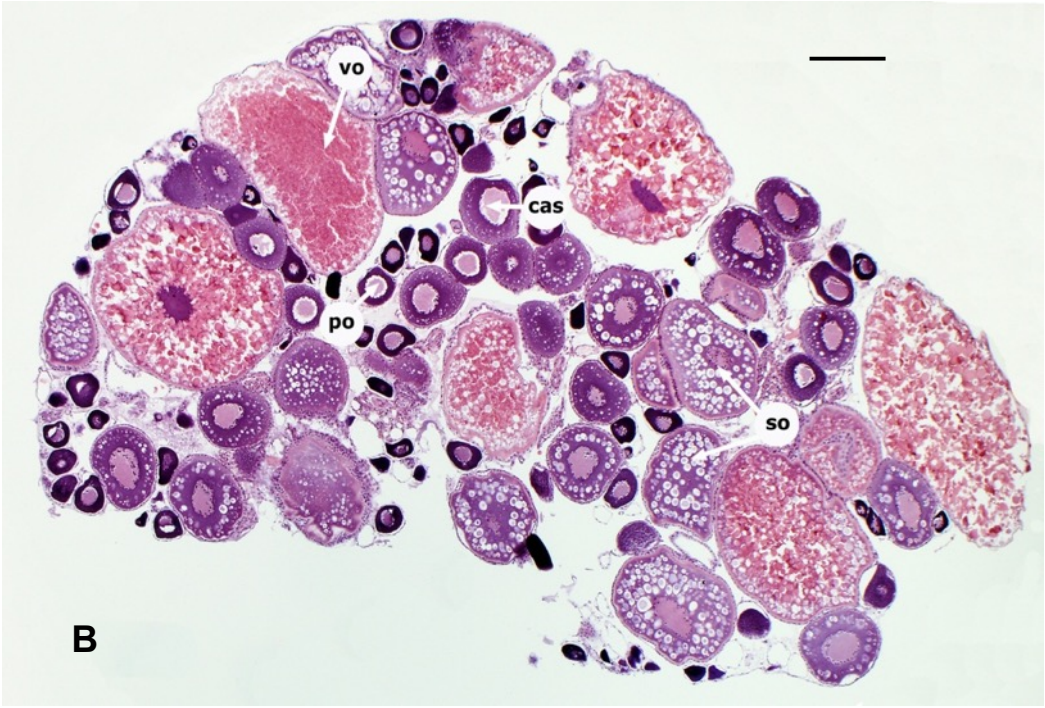
SI 3

SI 4 Water Chemistry LC-MS Quantification

Ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-26 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) maintained at 25 °C. Injection volume was 5 µL and mobile phase solvents were water (A) and acetonitrile (B) in an initial ratio (A:B) of 70:30. Separation was achieved at 25 °C using a flow rate of 0.25 mL/min with the following gradient: 70:30 to 30:70 in 8 min; 30:70 to 0:100 and held for 4 min; return to initial condition for 12 min and equilibration for 6 min. MS/MS was performed in the Multiple Reaction Mode (MRM) using ESI in the negative mode, and one characteristic fragment of the deprotonated molecular ion [M−H]− was used for quantitation. Other parameters were optimised as follows: capillary voltage −3.3 kV, extractor voltage 8 V, multiplier voltage 650 V, source temperature 120 ◦C, desolvation temperature 300 ◦C. Argon was used as collision 3 gas (P collision cell: 3×10−3 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 the oestrogen 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. Five point calibration curve (R2 > 0.99) covered the range 125–2500 pg (injected on column) for all compounds, within the linear range of the instrument.



(sg) spermatogonia (sc) spermatocytes (sz) spermatozoa (st) spermatids
Least advanced ◄——————————► Most advanced
Scale bar represent 20µm

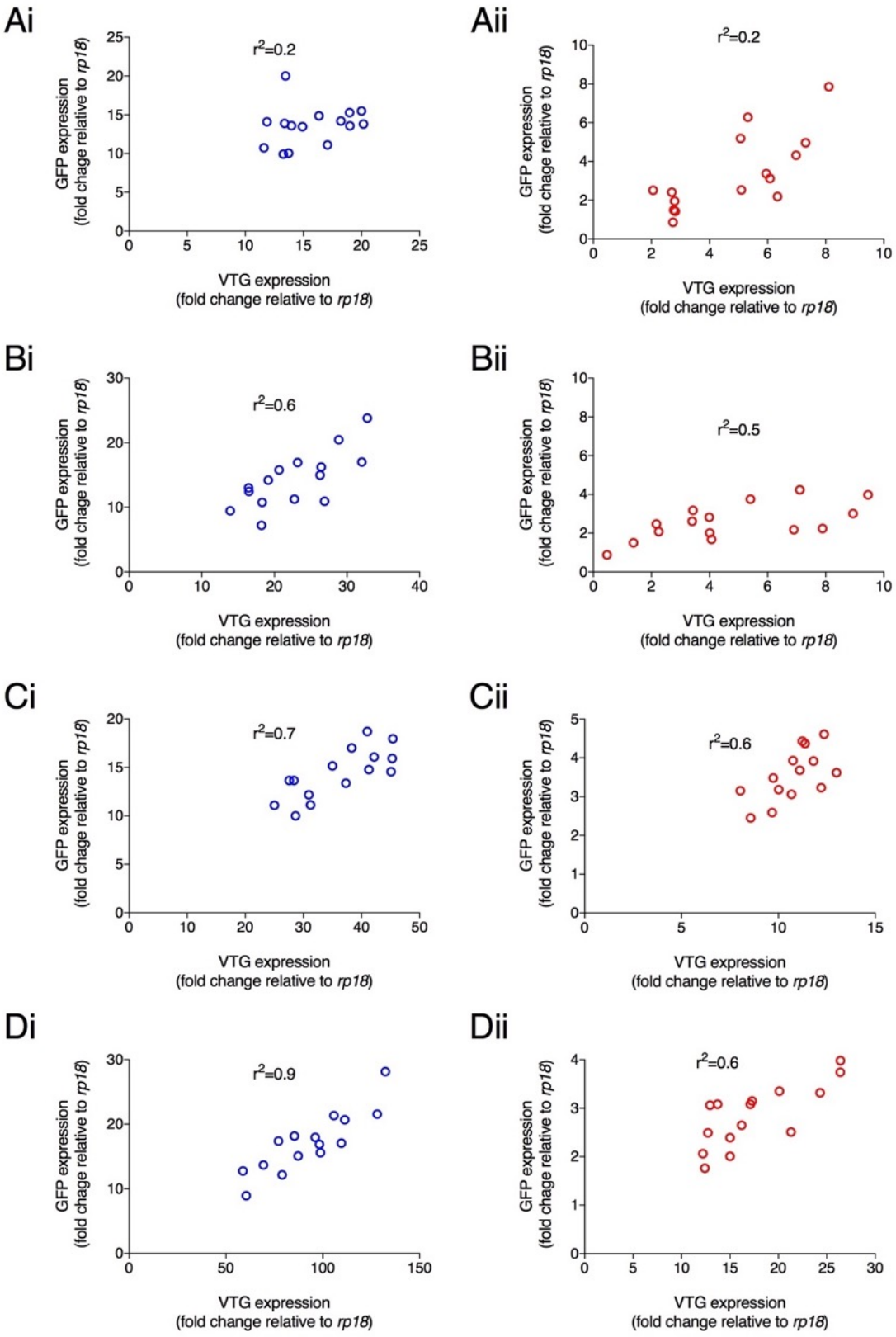


(po) primary oocyte (cas) cortical alveolous stage (so) secondary oocyte (vo) vitellogenic oocyte
Least advanced ◄——————————► Most advanced
Scale bar represent 100µm

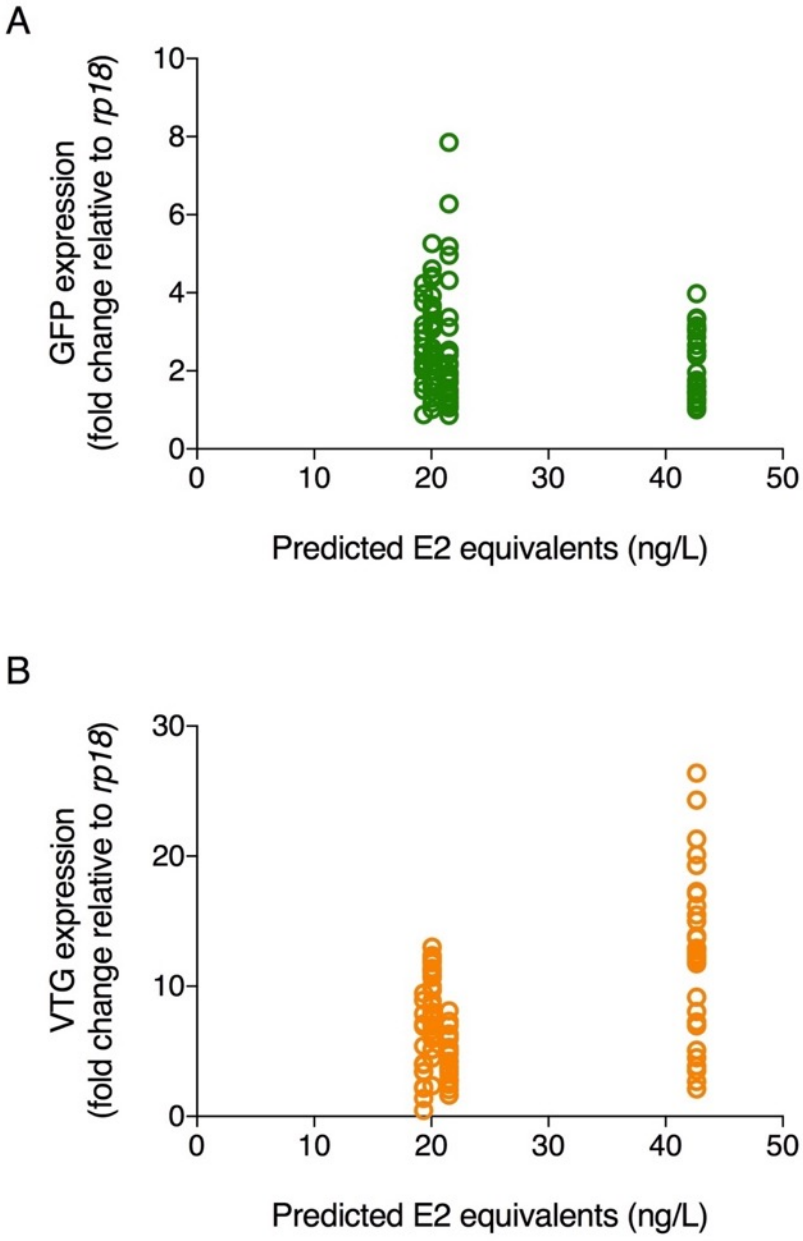
Sl 5a.

Ovary	Stages 0-5
	Stage 0 – Undeveloped - entirely immature phases (oogonia to perinucleolar oocytes); no cortical alveoli
	Stage 1 - Early spermatogenic - vast majority (e.g., > 90%) are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar.
	Stage 2 - Mid-development - at least half of observed follicles are early and mid-vitellogenic.
	Stage 3 - Late development - majority of developing follicles are late vitellogenic.
	Stage 4 - Late development/hydrated - majority of follicles are late vitellogenic and mature/spawning follicles; follicles are larger as compared to Stage 3.
	Stage 5 - Post-ovulatory - predominately spent follicles, remnants of theca externa and granulosa.
Testes	**Stages 0-4**
	Stage 0 – Undeveloped - entirely immature phases (spermatogonia to spermatids) with no spermatozoa.
	Stage 1 - Early spermatogenic - immature phases predominate, but spermatozoa may also be observed; the germinal epithelium is thinner than it is during Stage 2.
	Stage 2 - Mid-spermatogenic - spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions; the germinal epithelium is thinner than Stage 1, but thicker than Stage 3.
	Stage 3 - Late spermatogenic - all stages may be observed, however, mature sperm pre-dominate; the germinal epithelium is thinner than it is during Stage 2.
	Stage 4 – Spent - loose connective tissue with some remnant sperm.

SI 5b.


Ai
r²=0.2
GFP expression (fold chage relative to rp18)
VTG expression (fold change relative to rp18)
Aii
r²=0.2
GFP expression (fold chage relative to rp18)
VTG expression (fold change relative to rp18)
Bi
r²=0.6
GFP expression (fold chage relative to rp18)
VTG expression (fold change relative to rp18)
Bii
r²=0.5
GFP expression (fold chage relative to rp18)
VTG expression (fold change relative to rp18)
Ci
r²=0.7
GFP expression (fold chage relative to rp18)
VTG expression (fold change relative to rp18)
Cii
r²=0.6
GFP expression (fold chage relative to rp18)
VTG expression (fold change relative to rp18)
Di
r²=0.9
GFP expression (fold chage relative to rp18)
VTG expression (fold change relative to rp18)
Dii
r²=0.6
GFP expression (fold chage relative to rp18)
VTG expression (fold change relative to rp18)

SI 6.


A
GFP expression
(fold change relative to rp18)
10
8
6
4
2
0
0
10
20
30
40
50
Predicted E2 equivalents (ng/L)
B
VTG expression
(fold change relative to rp18)
30
20
10
0
0
10
20
30
40
50
Predicted E2 equivalents (ng/L)

SI 7.

Chapter 4.

Tissue and developmental responses to environmental oestrogenic pollutants assessed using an ERE-GFP transgenic zebrafish model

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4.1 Abstract

Oestrogen is essential for a range of developmental processes but exposure to exogenous chemicals with oestrogen mimicking effects has been correlated with various adverse health effects in both aquatic wildlife and in humans. Concern regarding exposure to oestrogenic endocrine disrupting chemcials has fueled international development of various screening tools, including transgenic fish models, for both risk assessment and to elucidate the mechanistic effect pathways and the potential for health impacts of these chemicals.

In this study, a novel oestrogen-responsive green fluorescent protein (ERE-GFP) Casper transgenic zebrafish model was applied to quantify tissue-specific responses to a suite of suspected environmental oestrogens during early life stages (0-4 days post fertlisation) vulnerable to developmental disruption. Measured endpoints included survival/hatching rate, GFP induction (measured in target tissues such as the liver via fluorescence microscopy, and via Western blotting or mRNA induction in whole embryos) and *vtg* mRNA induction. Lowest-observed-effect concentrations (LOECs) for *gfp* induction for the different environmental oestrogens were in the following order of magnitude: 0.001, 0.05, 10, 20, 100 $\mu g/L^{-1}$ (for EE_2, E_1, tamoxifen, nonylphenol, beta-sitosterol, respectively). Differences in tissue-specific patterns of GFP expression were observed between the different chemical exposures and revealed a novel life-stage specific agonistic behaviour of the pharmaceutical tamoxifen. Furthermore, when compared, GFP induction markers were similarly responsive to the measurement of *vtg* mRNA, a well-established biomarker for oestrogen exposure.

These findings further support the utility of biosensor transgenic zebrafish for assessing responses and effects for exposure to environmental oestrogens and illustrate the potential for differing health effects between different environmental oestrogens and even for a single oestrogenic compound over time.

4.2 Introduction

Endocrine disrupting chemicals (EDCs) are currently a major focal point of eco-toxicological research due to their widespread occurrence in the environment and capacity to disrupt normal endocrine system function in both humans and wildlife (Cristina *et al.,* 2012**;** Kabir *et al.,* 2015; Vethaak and Legler, 2013). These effects can be mediated through various mechanisms of action including via hormone receptor (ant)agonism, and /or alterations of hormone metabolism and/or synthesis and /or excretion (Kortenkamp *et al.,* 2011). EDCs encompass a diverse range of chemicals that include both natural steroid hormones, industrial chemicals (e.g. polychlorinated biphenyls [PCBs], phthalate plasticisers, alkylphenols), pesticides (including some herbicides and fungicides and organochlorine insecticides), pharmaceuticals (e.g. diethylstilboestrol [DES] and 17α-ethinyl oestradiol [EE_2]) and naturally occurring chemicals in plants (e.g. phytoestrogens).

The aquatic environment is particularly susceptible to the effects of EDCs, largely because it is a recipient for the vast majority of chemicals intentionally released into the environment (via effluent discharge from wastewater treatment works). This is evidenced by the fact that some of the best documented evidence of endocrine disruption in wildlife derives from studies on aquatic species, particularly freshwater fish. Effects include a range of reproductive and developmental alterations such as reduced sperm counts and motility, reduced fertility and an increased prevalence of intersexuality in fish (Bahamonde *et al.,* 2015; Brown *et al.,* 2008; Lange *et al.,* 2009; Niemuth and Klaper, 2015; Scholz and Kluver, 2009).

One of the most widely studied and perhaps most prevalent groups of EDCs are the environmental oestrogens, which encompass a diverse range of chemicals including the natural/synthetic steroidal oestrogens, (oestrone (E_1), 17β-oestradiol (E_2), oestriol (E_3) and EE_2 which account for the majority of oestrogenic activity in WwTW effluents (Jarosova *et al.,* 2014; Sumpter and Jobling 2013) as well as alkylphenols such as nonyphenol (NP), phytoestrogens (e.g. beta-sitosterol (BS)) and the plasticiser bisphenol A (BPA).

Some of these chemicals have structural similarities that enable them to interact with the ligand binding domain of the oestrogen receptor (ER) subtype, thus resulting in an interference of oestrogen regulated processes, whilst others alter normal ER signaling through multiple mechanisms, including protein-protein interactions with other transcription factors such as Fos/Jun i.e. AP-1-responsive elements. (Kusher *et al.,* 2000; Nelson and Habibi, 2013; Saville *et al.,* 2000) . Whilst the majority of environmental oestrogens found in the worlds waterways are typically present at levels well below concentrations often demonstrated to elicit observable health effects in fish in laboratory based assays, several studies have been published which provide compelling evidence to show that continuous, long-term exposure to specific environmental oestrogens (low sub ng/L to ng/L concentrations) can cause cumulative adverse reproductive health effects (in fish) which in some circumstances can lead to severe population level impacts (Kidd *et al.,* 2007; Lange *et al.,* 2011; Schwindt *et al.,* 2014). In addition, some environmental oestrogens such as EE_2 and NP have been shown to bio-accumulate in whole body tissues and the bile of fish (Al-Ansari *et al.,* 2010; Fenlon *et al,* 2010; Yang *et al.,* 2014) and in some circumstances may also biomagnify up the food chain (Cheng *et al.* 2006; Zenker *et al.,* 2014).

Many studies in mammals and human epidemiological research too, have shown that exposure to some environmental oestrogens may induce adverse reproductive and developmental health outcomes and also have wider health effects on the immune system, obesity and cardiovascular disease (Han *et al.,* 2016; Kuo *et al.,* 2012; Trasande *et al.,* 2012). As a result, international concern over the impacts of these chemicals on both human and environmental health has intensified significantly in the last decade, with many environmental oestrogens, including EE_2, BPA, NP and some phthalates, now listed internationally as priority substances.

Whilst studies have suggested associations between exposure to environmental oestrogens and the aforementioned diseases, these continue to be disputed, and there remains significant gaps in information which preclude the full establishment of the true impact of these chemicals on environmental and human health. These gaps in information include the effects of exposure to mixtures of chemicals, - particularly during early life stages and long term – and an

understanding of the underlying molecular mechanisms/modes of actions of oestrogen signalling, all of which are essential for assessing potential environmental and health risks.

Although there are several international programmes for the screening and testing of environmental oestrogens, most methods focus on a single interaction site (a chemical with a receptor, as in many *in vitro* systems), or on a single target tissue or effect outcome (e.g. enzyme-linked receptor assay) all of which are limited in their capacity for chemical metabolism, to calculate potency and elucidate tissue selectivity (Folmar *et al.,* 2002; Murk *et al.,* 2002).

Recently, transgenic biosensor fish (including zebrafish) have been developed that are responsive to oestrogens with their potential application to advancing our understanding on how environmental chemicals act within the body to affect health. In these genetically manipulated fish, which combine the complexity of a full organism with the ease and repeatability of a cellular assay, promoter elements encoding synthetic or natural oestrogen responsive elements (EREs) have been directly combined with reporter genes, such as luciferase or Green Fluorescent Protein (GFP) which are measured in body tissues signaling for tissue repsonses. (Brion *et al.*, 2012, Chen *et al.,* 2010, Ji *et al.,* 2012, Lee *et al.,* 2012, Gorelick *et al.,* 2014; Green *et al.,* 2016).

Preliminary work using a novel, ERE-GFP-Casper Zebrafish model, developed at the University of Exeter (Green *et al.,* 2016) , has shown it to have high sensitivity to oestrogen (including for environmentally relevant concentrations) detecting multiple tissue responses for oestrogenic (GFP) signaling (liver, heart, brain, forebrain, otic vesicle, neuromast, gut and muscle), consistent with both the known expression patterns (collectively) for the different ER subtypes and some other ERRs in fish (and mammals) and linked with the very diverse roles of oestrogens in vertebrate function. The lack of skin pigmentation in the Casper ERE-TG model also allows for more effective detection of the fluorescent signal in deeper body tissue compared with pigmented transgenic lines.

The aim of this study was to further assess the sensitivity and repeatability of the ERE-GFP-Casper zebrafish system for determining whole body responses to environmental oestrogens and for characterising the tissue specificities of oestrogenic chemicals to inform on target tissues and potential health outcomes. To this end a series of known EDCs, which have been identified or suspected of having oestrogenic properties were selected, including the natural steroidal oestrogen, oestrone (E_1); the synthetic oestrogen 17α-ethinyl estradiol (EE_2) which is widely used in the contraceptive pill and in hormone replacement therapy; the pharmaceutical tamoxifen (TAM), used to treat and prevent some types of breast cancer; the plant sterol beta-sitosterol (BS); 4-nonylphenol (NP), a common industrial surfactant; and perfluorooctanoic acid (PFOA), used extensively in the manufacture of water and stain resistant products (see SI 1 for chemical structures). All of these chemicals have been associated with detrimental health effects in biota and humans and are prevalent in the aquatic environment.

A further aim of this study was to establish how the ERE-GFP-Casper zebrafish model compared, in terms of sensitivity for detecting oestrogenic responses with *vtg* production in fish, a well established and sensitive biomarker for exposure to oestrogenic chemicals.

4.3 Materials and methods

4.3.1 Test Organisms. A transgenic zebrafish developed at the University of Exeter, ERE-GFP-Casper (3×ERE:Gal4ff and UAS:GFP) was employed for this study. The ERE-GFP-Casper line was derived from crossing an established transgenic ERE-GFP line (Lee et al., 2012) with a Casper strain acquired from University College London (see **SI 1** for full details). All zebrafish were raised at 28 °C under a 12-h light, 12-h dark cycle photoperiod regime, fed to satiation three times daily with ZM Fry food 000 (Zebrafish Management Ltd., Wincester, U.K.) and Liquifry No. 1 (Interpet, Dorking, U.K.).

4.3.2 Experimental design. ERE-GFP-Casper zebra fish embryos (*n*= 50 per treatment x 3 replicates held in 400ml sterile glass dishes) were exposed from fertilisation to 96 hours post-fertilisation (96hpf) to either a dilution water control (DWC) or a suspected environmental estrogen, at a series of exposure concentrations as follows: EE_2 (1, 5, 10 ng/L); E_1 (10, 50, 100 ng/L); NP (10, 20, 100 µg /L); Tamoxifen (10, 20, 100 µg /L); BS (100, 200, 1000 µg /L); PFOA (100, 200, 1000 µg /L). Stock solutions of the different chemicals (purchased from Sigma, Poole, UK) were prepared in acetone (10 mg/L) and stored in a dark cold-room (4 °C) to minimise the risk of degradation. Solvent free working solutions were prepared (in embryo culture water) the day before use, by pipetting the required amount of stock solution into a glass duran bottle and evaporating the solvent away under a stream of nitrogen. In the embryos cultures, 50% of the treatment medium was replaced every 24 hours and all treatments were gently aerated using a glass pipette connected to tubing fed through each dish lid to ensure that the dissolved oxygen concentration remained greater than 80% of the air saturation value.

4.3.3 Fish sampling and analysis of phenotypic endpoints. All animal use protocols were carried out ethically in accordance with U.K. Home Office guidelines (Animals (Scientific Procedures) Act 1986). Fish were sacrificed by terminal anesthesia with benzocaine (0.5 g/L; ethyl-*p*-aminobenzoate; Sigma, Poole, U.K.). For each chemical exposure, triplicate sets of embryos were sampled at 96 hpf and examined for GFP expression using Western blot analysis (*n*= 10 fish per treatment) and image analysis (*n*= 15 fish per treatment) and for

VTG/GFP gene expression analysis (*n*= 15 fish per treatment). Other measured endpoints include survival and hatching rate (success).

4.3.4 Determination of chemical concentration in exposure water. To assess the concentrations of chemicals in the exposure water in the glass dishes, nominal aqueous exposure concentrations were tested at day 0 and day 4 of exposure (i.e. 0 and 4 dpf). Control, minimum and maximum concentrations were sampled for each chemical. For all chemicals, with exception of EE_2, water samples were diluted in acetonitrile (ACN) before analysis by tandem liquid chromatography-mass spectrometry (LC-MS). Due to the low concentration of EE_2, samples were initially run through solid phase extraction (SPE) cartridges (Sep-Pak Plus C18) into ACN, to achieve a detectable concentration for LC-MS analysis. See Supporting Information for full details of LC-MS analysis.

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

4.3.6 Quantification of GFP (EGFP) expression using western blot analysis. All fish were sampled at 96hpf to quantify whole body GFP expression using Western blot analysis, according to the protocol described previously (Lee *et al.,* 2012), with the strength of the GFP signal evaluated with Image J software (http://rsbweb.nih.gov/ij/) standardized to the intensity of alpha tubulin and shown as a fold increase in GFP levels in exposed embryos over unexposed embryos.

4.3.7 Quantification of the levels of GFP mRNA and VTG mRNA in whole embryo-larvae using RT-qPCR.

4.3.7.1 RNA extraction and cDNA synthesis.

Samples for measurement of whole body VTG and GFP mRNA were immediately snap-frozen in liquid nitrogen upon collection and stored at −80°C until use. Total RNA was extracted from all samples using Trizol Reagent (Sigma), according to the manufacturer's instructions. The amount of RNA was quantified using a NanoDrop 1000 spectrophotometer and RNA purity determined through the measurement of the A260/A280 and A260/230 ratios, respectively. Reverse transcription was subsequently carried out by incubating 1ug RQ1 DNase treated (Promega) total RNA with 5 mM random hexamers (Eurofins MWG Operon) 10 mM dNTPs and 1ul MMLV-Reverse transcriptase (Promega) in the appropriate buffer for 60 min at 37 °C and 10 min at 42 °C. Polymerase chain reaction (PCR) was performed in an iCycler hermocycler coupled to a MyiQ detector (Bio-Rad. Hercules, CA, USA).

*4.3.7.2 Real-time quantitative PCR***.**

RT-qPCR was carried out on cDNA samples for each treatment group and sampling point for selected target genes with the iCycler iQ Real-time Detection System (Bio-Rad Laboratories Inc., CA, USA) using the following primers: Vtg1 (fw) 5'-GCCAAGAAAGAACCCAAACTG-3', Vtg1 (rev) 5'-GGAGATGAGAGCCACTGAAG-3'; EGFP (fw) 5'-CGACGGCAACTACAAGAC-3', EGFP (rev) 5'-TAGTTGTACTCCAGCTTGTGC -3'. Primer sequences were either designed with Beacon Designer software (Premier Biosoft International, Palo Alto, USA) or using previously published sequences (Brion et al., 2012), and obtained from MWG-Biotech (Ebersburg, Germany). In brief, primer pairs were optimized for annealing temperature (Ta), specificity confirmed by melt curve analysis, and the detection range, linearity and amplification efficiency (*E*) established using serial dilutions of zebrafish cDNA. RT-qPCR was carried out using Absolute QPCR SYBR Green Fluorescein mix (ABgene), with an initial activation step of 95 °C for 15 min followed by 40 cycles of denaturation (95 °C, 10 s) and annealing (appropriate Ta, 45 s) and final melt curve analysis. Expression levels of ribosomal protein L8 *(rpl8)* were used as a 'housekeeping' gene to normalize the expression of other genes using a development of

efficiency correlated relative quantification as described previously (Filby and Tyler, 2005).

4.3.8 Statistical analysis. In all cases, data were checked for normality using the D'Agostino-Pearson test and for homogeneity of variance using Bartlett's or Levene's test prior to statistical analysis. Data meeting these tests were analysed using parametric tests, e.g. analysis of variance (ANOVA) procedures, whilst data that did not conform to normality were subject to nonparametric tests, e.g. Kruskal–Wallis. Post hoc analysis was performed against controls using the Dunnett's test and with the Tukey test for between-treatment comparisons. Data were transformed, where necessary, using log10. Any significant differences from expected sex ratios or stages of gonadal development were tested using the chi-square test. All statistical analyses were run in GraphPad Prism 7 (GraphPad Software, Inc., San Diego, USA). Statistical significance was accepted as $p < 0.05$ for all comparisons and values are quoted as mean values and ranges as standard error of the mean.

4.4 Results

4.4.1 Water Chemistry Analysis

Measurable concentration values for all test chemicals in all control samples were less than the limit of quantitation (LOQ).

For EE_2, E_1 and tamoxifen, measured concentrations were highly consistent and within 20% of day 4 nominals across the concentration ranges tested (see Table 1). For EE_2, mean measured concentrations were 1.24 ng EE_2 /L^{-1}, 6 ng EE_2 /$L^{-}1$ and 9.7 ng EE_2 /L^{-1} (nominals 1, 5, and 10 ng/L), for E_1, mean measured concentrations were 6.3 ng E_1 /L^{-1}, 51.3 ng E_1 /$L^{-}1$ and 95.9 ng E_1 /L^{-1} (nominals 10, 50, and 100 ng/L), and for tamoxifen mean measured concentrations were 10.4 µg TAM/L^{-1}, 25.6 µg TAM /$L^{-}1$ and 102.4 µg TAM /L^{-1} (nominals 10, 20, and 100 µg/L). Measured concentrations for NP were somewhat lower than nominal. For the highest NP exposure concentration (nominal 100 µg/L), the measured concentration was 62% of nominal on day 0, and 40% on day 4, and for the lower exposure (nominal 10 µg /L) the measured concentrations were 65% and 51% of nominal on days 0 and 4, respectively (**Table 1**).

Chemical analysis for both BS and PFOA was not carried out.

Test substance	Nominal concentrations	Measured concentrations	
		Day 0	Day 4
17α-ethinyl estradiol	1, 5 and 10ng/L	1.38ng/L (138%) 6.2ng/L (124%) 10.3ng/L (103%)	1.1ng/L (110%) 5.8ng/L (116%) 9.2ng/L (92%)
Estrone	10, 50 and 100ng/L	12.3ng/L (123%) 58.4ng/L (117%) 110.9ng/L (111%)	8.3ng/L (83%) 44.2ng/L (88%) 80.9ng/L (89%)
4-nonylphenol	10, 20 and 100µg/L	6.5µg/L (65%) 12.3µg/L (62%) 61.7 µg/L (62%)	5.1µg/L (51%) 11.4µg/L (57%) 40.3µg/L (40%)
Tamoxifen	10, 20 and 100µg/L	11.6µg/L (116%) 28.1µg/L (140%) 114.2µg/L (114%)	9.2µg/L (92%) 23.1µg/L (115%) 90.7µg/L (91%)
Beta-sitosterol	100, 200 and 1000µg/L	*dna*	*dna*
Perfluorooctanoic acid	100, 200 and 1000µg/L	*dna*	*dna*

dna = data not available.

Table 1. Measured water concentrations for each of the oestrogenic chemicals taken on day 0 and day 4. Data as shown are the mean of 3 replicate exposures, repeated 3 times.

4.4.2 Phenotypic endpoints

4.4.2.1 Embryo survival and hatching success

There was no significant effect on survival in any of the treatment groups for EE_2, NP, tamoxifen or BS (**Fig 1 A; C; D; E**). For E_1, survival was affected only by exposure to 100 ng/l at 24, 48, 72 and 96 hpf (65/90 = 72%, p =<0.01, 64/90 = 71%, p=<0.01, 54/90 = 66%, p=<0.01, respectively, **Fig 1B**). There was also a significant difference in the survival rate of those fish exposed to 1000 µg/l PFOA through all time points (67/90 = 74%, p =<0.01, 62/90 = 69%, p=<0.01, respectively, **Fig 1F**).

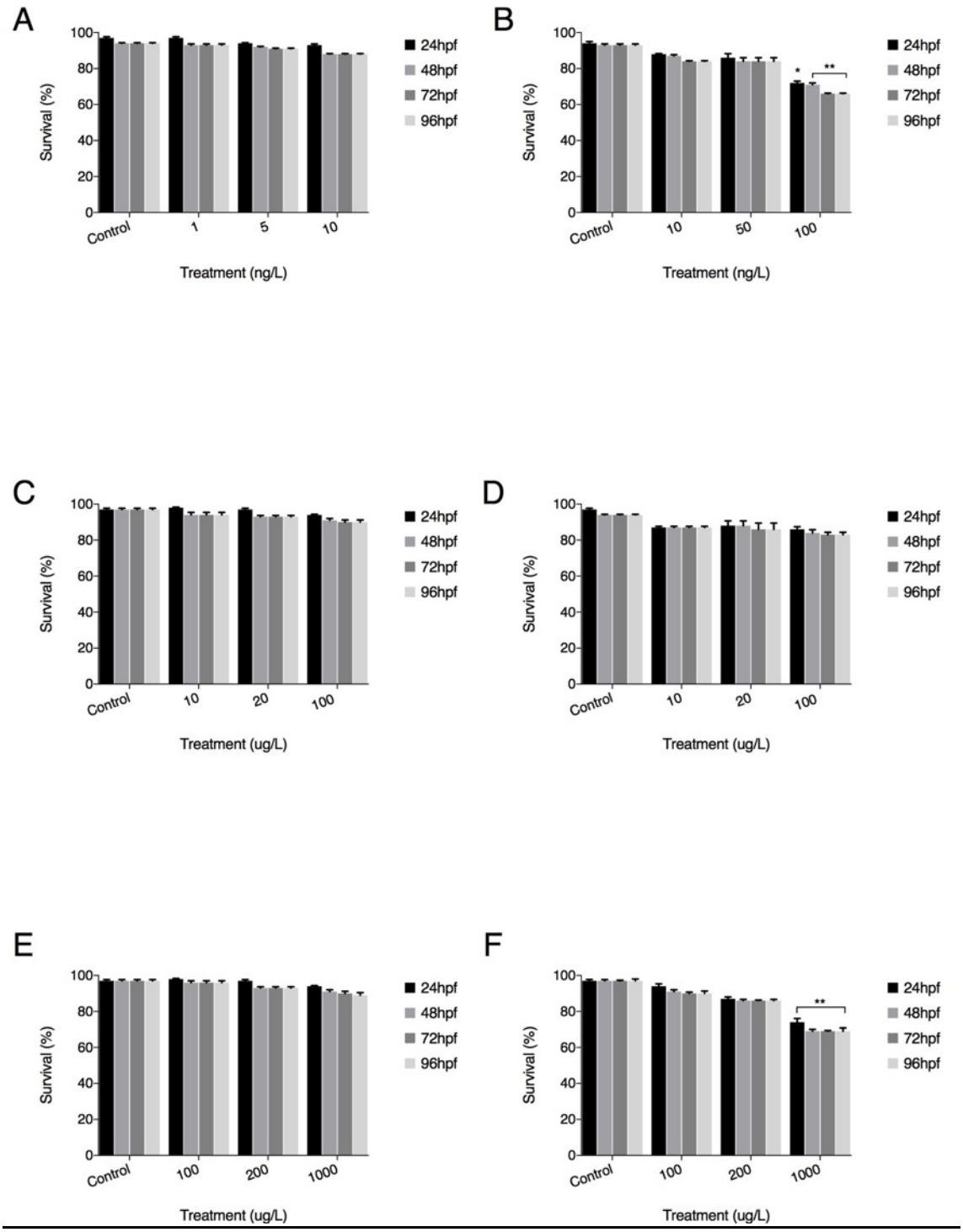

Figure 1. Survivorship analysis of ERE-GFP Casper zebrafish exposed to various environmental oestrogens at from fertilisation to 96 hours post fertilisation. Transgenic zebrafish embryos/ were exposed to EE_2 (A), E_1 (B), NP (C), TAM (D), BS (E), PFOA (F), all with dilution water controls. Data are reported as mean ± SEM. Asterisks denote a significant difference (**p<0.01) compared with controls.

As all surviving embryos in control groups across all treatments had hatched by 72hpf, and this timepoint was subsequently used to compare with hatching success in other treatments groups. There was no significant difference in hatching at 72 or 96hpf, in any of the treatment groups for NP, tamoxifen, BS, or PFOA (**Fig. 2C; D; E; F**). Hatching success in E_1 treatments were significantly delayed in 10 and 50 ng/l exposed embryos at 72hpf (63/72 = 87%, *p* =<0.01, 62/76 =81%, respectively, **Fig 2B**). For EE_2 exposures, hatching success was significantly delayed at 72hpf in 10 ng/l exposed embryos only (59/79 = 75%, *p* =<0.01, **Fig 2A**).

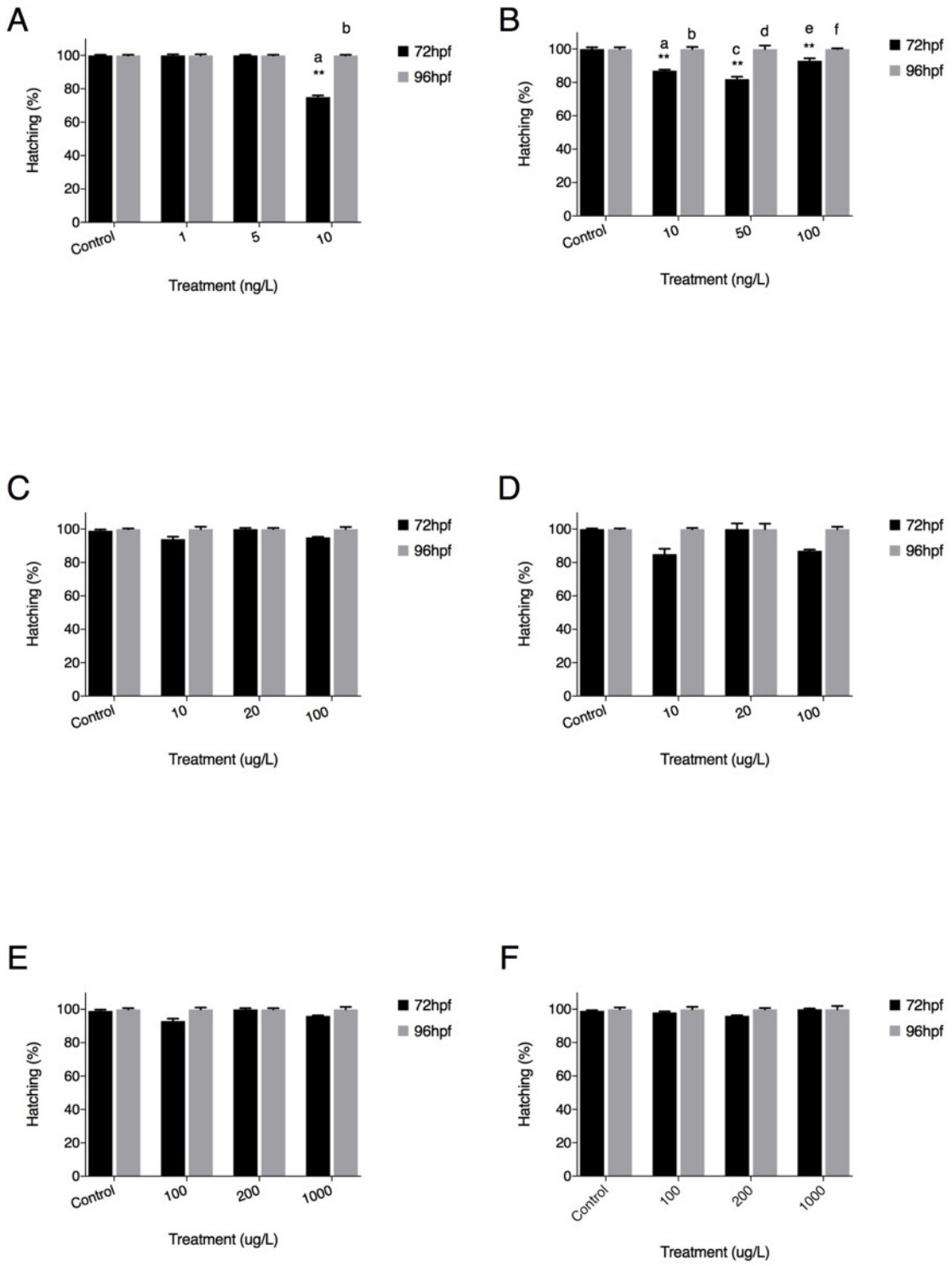

Figure 2. Hatching rates in ERE-GFP Casper zebrafish exposed to various environmental oestrogens. Transgenic zebrafish embryos were exposed to EE_2 (A), E_1 (B), NP (C), TAM (D), BS (E) or PFOA (F) all with dilution water controls. Data are reported as mean ± SEM. Asterisks denote a significant difference (**p<0.01) compared with control. Different letters denote a significant difference within the treatment groups (p<0.05).

4.4.2.2 GFP expression

Analysis of confocal images of the ERE-TG Casper zebrafish, demonstrated that exposure to different chemicals induced different responses in GFP induction, in terms of both tissue type patterns and in GFP intensity (**Fig.3**). Control larvae across all exposures showed a weak basal GFP expression in both the heart, (with a 47% frequency in the larval population), and in the otic vesicle, (54% of the larval population) at 96hpf. Auto-fluorescence was also observed in the yolk sack. (**Fig. 3Ai-iii**).

In larvae exposed to E_1, GFP was induced in the heart, liver, somites, otic vesicle, forebrain, cranial muscles, neuromasts, and in the pectoral fin with levels of GFP expression appearing to increase in a concentration-dependent manner (**Fig. 3Ci-iii; Fig 4E-G**). GFP tissue expression patterns induced by EE_2 were similar to that of E_1, where responses were titrated down to 1 ng/L, with low level GFP expression induced in cells in the liver and in the somites (**Fig. 4Di-iii**). Again, higher exposure concentrations resulted in increasingly higher GFP expression levels, and at 10 ng/L EE_2 was observed throughout the whole liver, together with other GFP expressed domains in the heart, forebrain, somites (anterior and posterior), fin, cranial muscle and in the otic vesicle (**Fig. 3Bi-iii**).

In contrast with the responses to the steroidal oestrogens EE_2 and E_2, the lowest-observed-effect concentrations (LOECs) for NP, tamoxifen and BS responses were at relatively high exposure concentrations (10 µg/L, 10 µg /L, and 100 µg /L, respectively). GFP tissue expression patterns induced by tamoxifen and NP were similar to that of EE_2, (**Fig. 3D-E**), with additional GFP expression observed in the pectoral fin in larvae exposed to 100 ug/L tamoxifen (**Fig.3Ei**). In larvae exposed to BS, GFP expression was observed in the heart, ov ganglions, retinal ganglions, forebrain and otic vesicle (**Fig. 3Fi-iii**), with little or no GFP expression seen in the somites and liver respectively. The LOEC for this chemical was 100 µg /L. For all PFOA exposures, GFP expression was observed in the otic vesicle only (**Fig. GFi**).

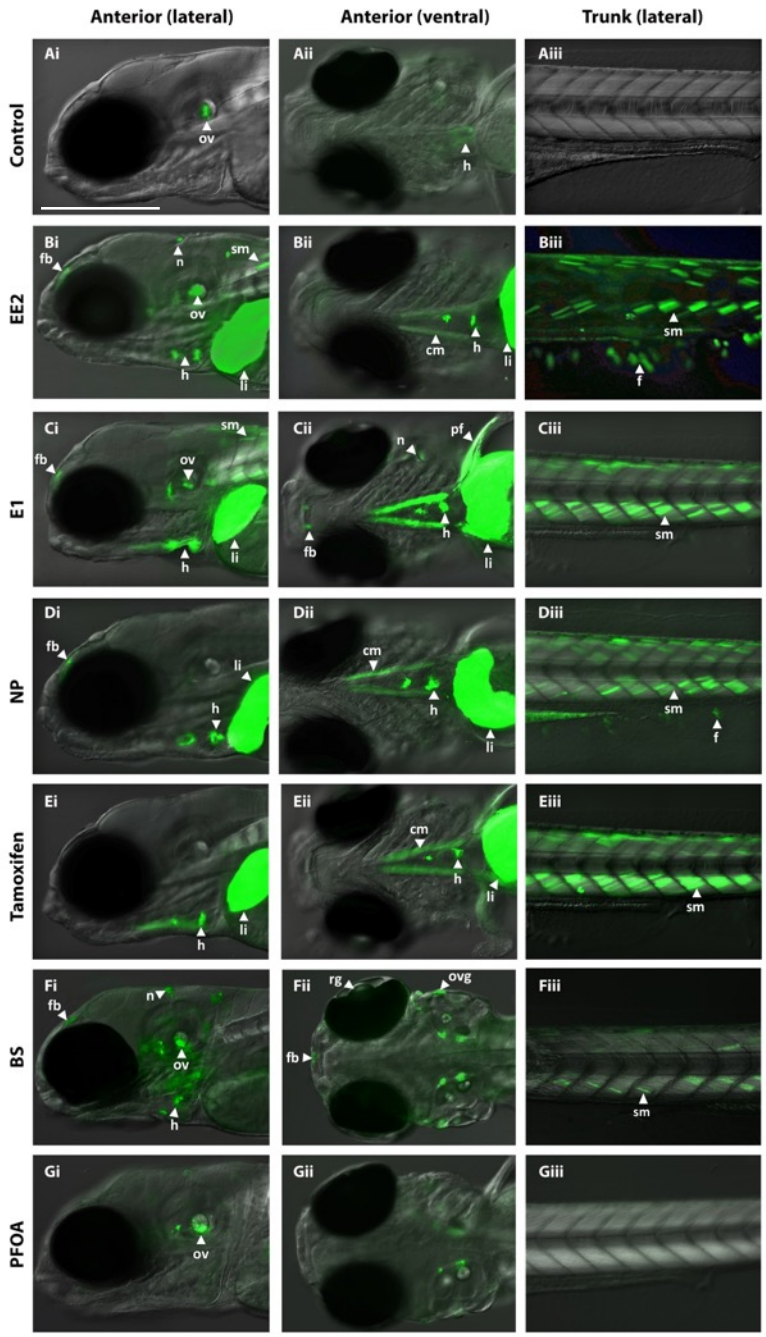

Figure 3. Expression of GFP in body tissues in ERE-GFP Casper zebrafish exposed to various suspected oestrogenic chemicals. Transgenic zebrafish embryos were exposed to dilution water (**A**), EE_2 (10 ng/L) (**B**), E_1 (100 ng/L) (**C**), NP (100μg/L) (**D**), TAM (100μg/L) (**E**), BS (1000μg/L) (**F**), and PFOA (1000μg/L) (**G**). GFP expression is shown for regions in the head (lateral – i and ventral – ii views) and trunk (lateral view - iii). In controls GFP induction was observed in the otic vesicle (ov), and weakly soin the heart (h) (**A**). The different oestrogenic chemicals induced different tissue patterns of GFP expression (**B-G**). EE_2 (**B**) and E_1 (**C**), induced GFP expression in the liver (li), forebrain (fb), cranial muscles (cm), fin (f), neuromasts (n), pectoral fin (pf), and somite muscles (sm). For NP (**D**) and TAM (**E**) exposed larvae GFP was enhanced in the h, li, ov, fb and sm. In BS-exposed larvae (**F**), GFP expression was detected in the ov ganglions (ovg), retinal ganglions (rg), fb, h, ov, n, and sm. In PFOA-exposed larvae (**G**) induced GFP expression in the ov only. Bar = 200 μm.

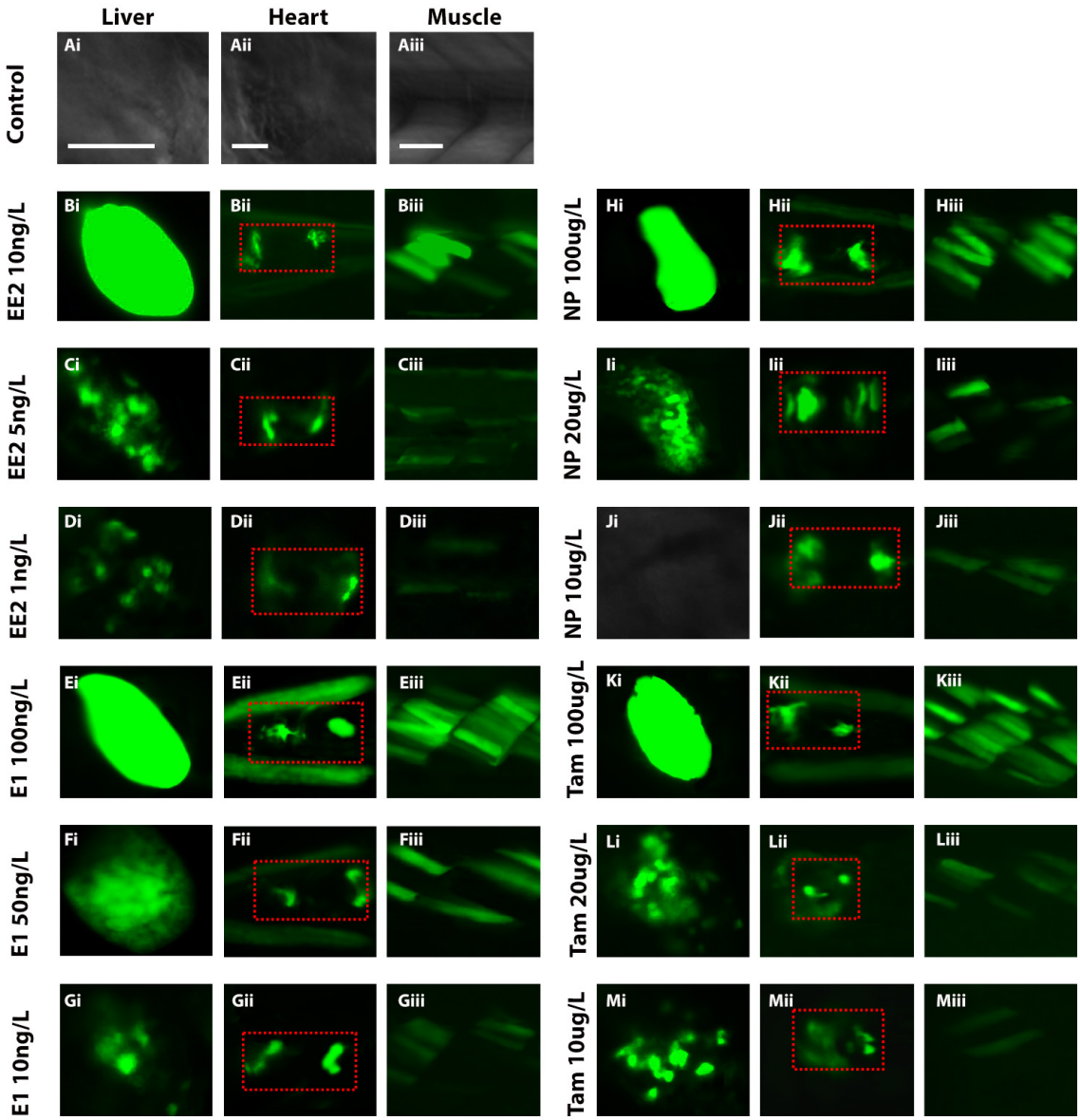

Figure 4. Expression of GFP in the liver, heart and somite muscle in whole ERE-GFP Casper zebrafish exposed to selected oestrogenic chemicals from fertilisation to 96 hours post fertilisation. Transgenic zebrafish embryos at 4dpf in unexposed controls (**A**), EE_2 (1, 5, 10 ng/L) (**B**), E_1 (10, 50, 100ng/L) (**C**), NP (10, 20, 100μg/L) (**D**), TAM (10, 20, 100μg/L) (**E**), BS (100, 200, 1000μg/L) and PFOA (100, 200, 1000μg/L) (**G**). GFP expression is shown in lateral view in the liver (i) and somite muscle (iii), and in ventral view in the heart (ii). Bars = 100 μm.

Analysis of the response dynamics during early life development (from 24hpf to 96hpf) were assesed for exposures to EE_2 and tamoxifen (10 ng/L and 100 ug/L respectively) (**Fig. 5**). Expression of GFP in larvae exposed to EE_2 was initially seen at 24 hpf in the heart, liver, and muscle, with the levels increasing gradually to 96 hpf (**Fig. 5A**). In comparison, the induction of GFP for exposure to EE_2 in the nervous system occurred later in development; GFP in the otic vesicle was first observed after 48 hpf, and subsequently in the forebrain at 72hpf.

For tamoxifen exposures, the ontogeny of GFP expression in the different tissue types was comparable to that of EE_2, in the liver, muscle and heart (24hpf). However, induction of GFP in the nervous system was different to EE_2, with an earlier response in both the otic vesicle (24hpf) and forebrain (48hpf) (**Fig. 5B**).

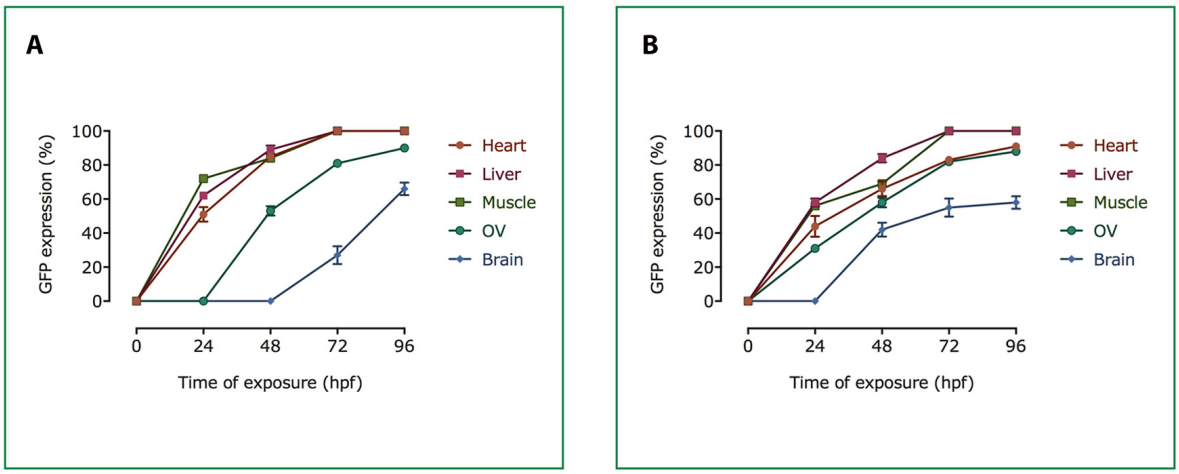

Fig. 5 Time related analysis of GFP expression. Data shows the perectage of ERE-GFP Casper embryos (n=45) expressing GFP in the heart, liver, muscle, otic vesicle and brain. Data are reported as mean ± SEM.

In general, levels of GFP expression appeared to be positively associated with the concentration of the exposure, as quantified via western blotting (**Fig. 6**).

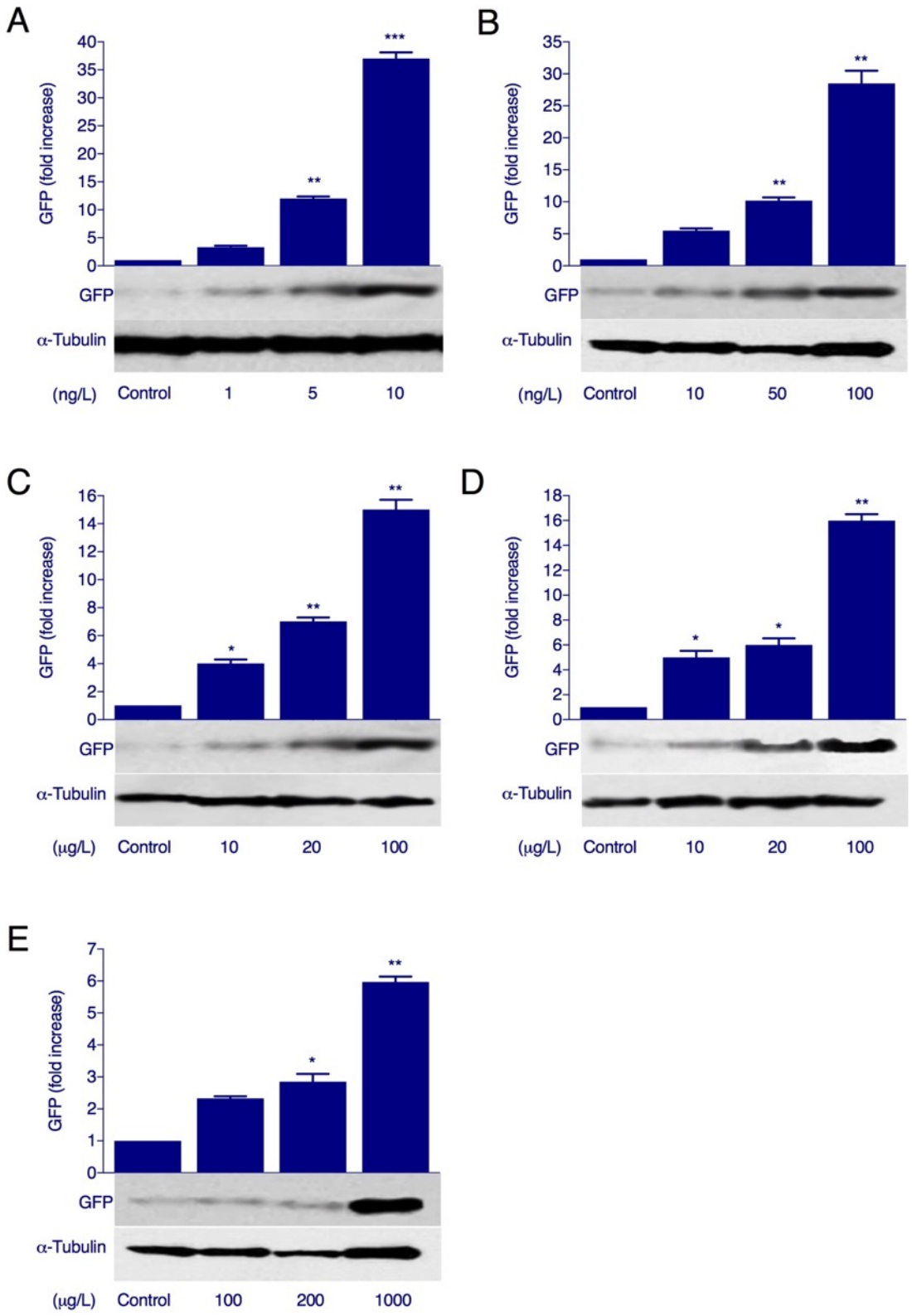

Figure 6. GFP expression quantified through Western blotting. Data shows concentration response for GFP induction for exposures to EE_2 (A), E_1 (B), NP (C), Tamoxifen (D), and BS (E). Analysis was conducted three times and data are reported as mean ± SEM. α-Tubulin was used as a loading control. Asterisks denote a significant difference (*p<0.05, **p<0.01) compared with control.

4.4.2.3 VTG/GFP mRNA expression

A significant induction on *gfp* mRNA induction was seen for exposures to 5 and 10 ng EE_2 $/L^{-1}$ when compared with untreated controls (2.8-fold induction, p=<0.05, and 9-fold induction, p=<0.01, respectively, **Fig. 7A**). For E_1 exposures induction was observed in 50 and 100 ng E_1 $/L^{-1}$ exposed embryos (4.7-fold induction, p=<0.001, and 7.8-fold induction, p=<0.001, respectively, **Fig. 7B**). For NP *gfp* mRNA induction was significantly upregulated in both 20 and 100 µg NP $/L^{-}$ (5.8-fold induction, p=<0.05, and 8-fold induction, p=<0.001, respectively, **Fig. 7C**). A significant *gfp* mRNA induction was observed in 20 µg and 100 µg exposures for TAM $/L^{-1}$ treatment groups only (4.5-fold induction, p=<0.0001, and 5.8-fold induction, p=<0.0001, respectively, **Fig. 7D**). For both BS and PFOA exposed embryos, although there appeared to be an induction of both *gfp* mRNA in 1000 µg /L treatments (2 to 3-fold high compared to controls) these elevations were not found to be statistically significant (**Fig. 7E-F**).

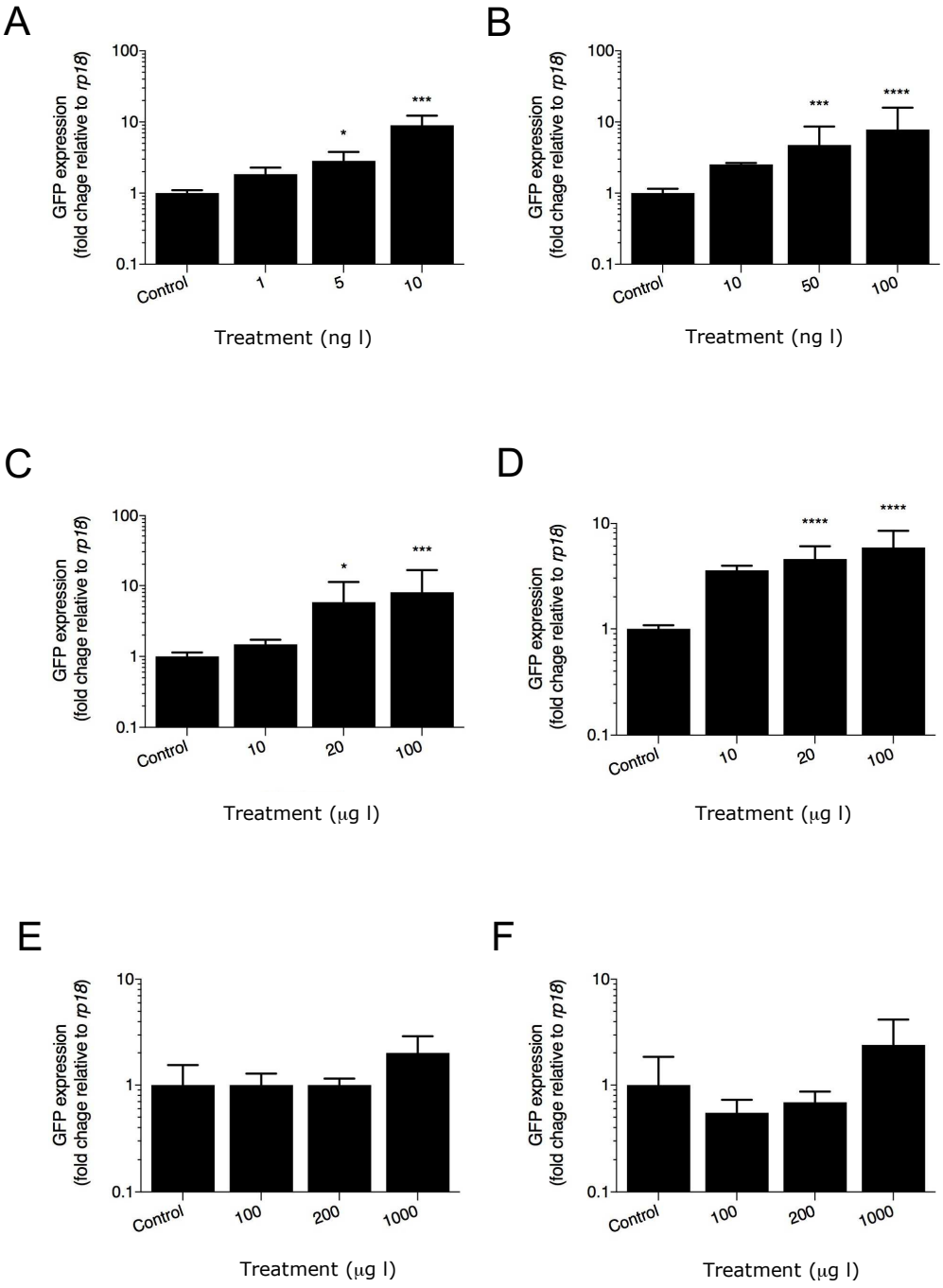

Figure 7. GFP mRNA expression in ERE-GFP Casper zebrafish. Transgenic zebrafish were exposed from fertilisation to 96 hours post fertilisation to EE_2 (**A**), E_1 (**B**), NP (**C**), TAM (**D**), BS (**E**) and PFOA (**F**). Data are reported as mean ± SEM and expressed as fold change compared with the control. Relative mRNA expression was determined as the ratio of target gene mRNA/rpl8 mRNA. Asterisks denote a significant difference (*p<0.05, **p<0.01) compared with controls. Within groups, different letters denote a significant difference (p<0.05).

Quantification of *vtg* mRNA in whole embryos exposed to EE_2 showed a significant induction for all exposures treatments (1 ng EE_2 /L^{-1}, 3.1-fold induction, p=<0.05; 5 ng EE_2 /L^{-1}, 12.8-fold induction, p=<0.01; 10 ng EE_2 /L^{-1}, 46.5-fold

induction, p=<0.001, respectively, **Fig. 8A**). For E_1 exposures there was a significant effect on *vtg* mRNA induction in embryos exposed to all treatments (10 ng E_1 /L^{-1}, 5.6-fold induction, p=<0.05; 50 ng E_1 /L^{-1}, 13.4-fold induction, p=<0.01; 100 ng E_1 /L^{-1}, 39.7-fold induction, p=<0.001, respectively, **Fig. 8B**). Induction of *vtg* mRNA occurred in embryos following exposure to 100 µg NP /L^{-1}, compared to untreated controls (15.2-fold induction, p=<0.01, **Fig. 8C**). For tamoxifen, a significant effect on *vtg* mRNA induction was observed for both 20 µg TAM /L^{-1} and 100 µg TAM /L^{-1} treatments (9.6-fold induction, p=<0.01; 18.4-fold induction, p=<0.001, respectively, **Fig. 8D**). For both BS and PFOA exposed embryos, although there appeared to be an induction of *vtg* mRNA in 1000 µg /L treatments (2 to 3-fold high compared to controls) these elevations were not found to be statistically significant (**Fig. 8E-F**).

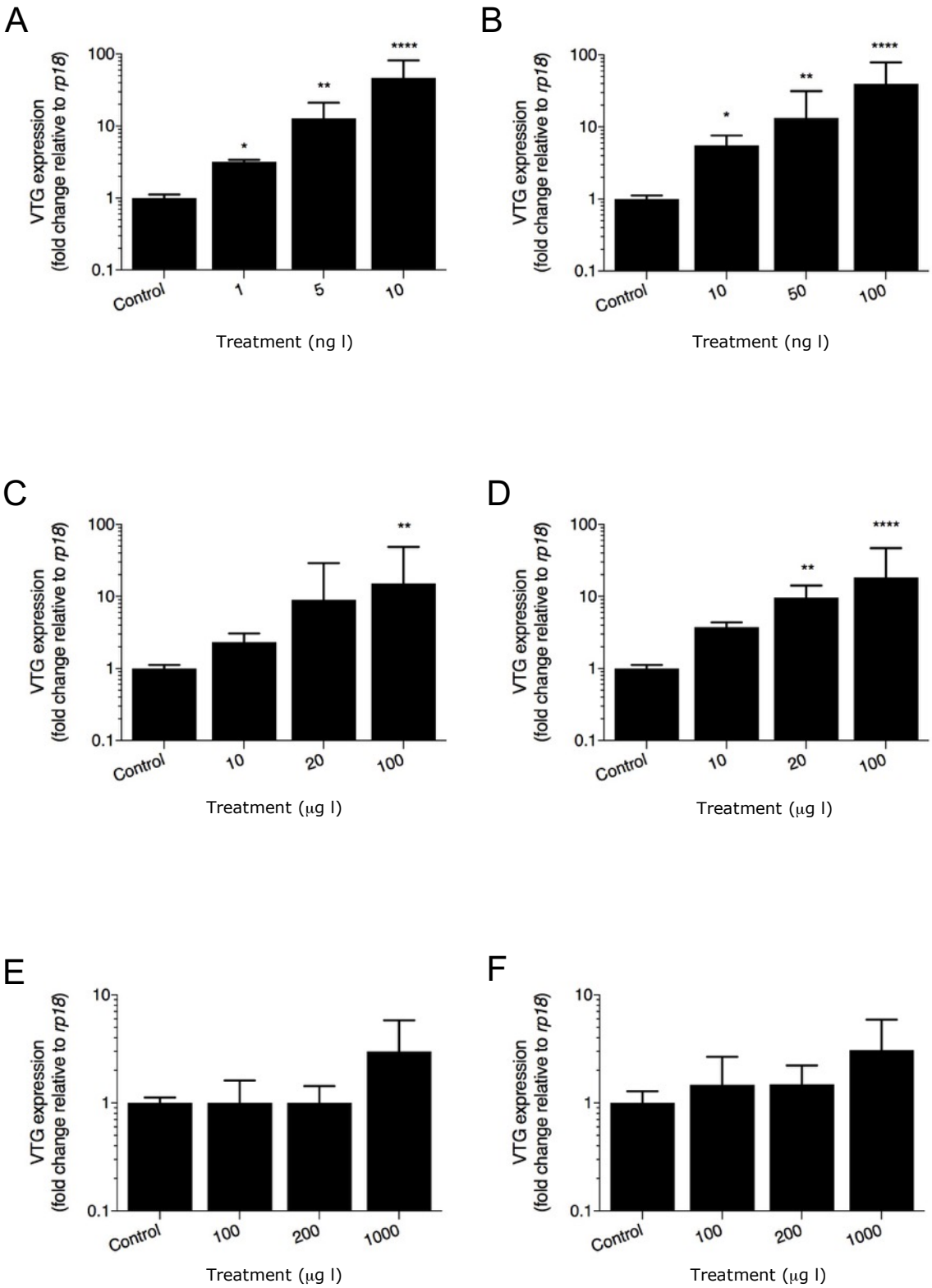

Figure 8. VTG mRNA expression in ERE-GFP Casper zebrafish. Data show fold change in VTG mRNA compared with controls following exposure to EE_2 (**A**), E_1 (**B**), NP (**C**), TAM (**D**), BS (**E**) and PFOA (**F**). Data are reported as mean ± SEM. Relative mRNA expression was determined as the ratio of target gene mRNA/rpl8 mRNA. Asterisks denote a significant difference (*p<0.05, **p<0.01) compared with control. Within groups, different letters denote a significant difference (p<0.05).

A comparison of the induction levels of *gfp* mRNA against *vtg* mRNA in whole embryo homogenates showed that the magnitude for responses in *vtg* induction was higher compared to *gfp* for all treatments, with the exception for BS and PFOA (Fig 9). Comparison of the data as a whole revealed a positive correlation between the levels of *gfp* expression and the levels of *vtg* expression to varying degrees between the different chemicals. For BS and PFOA, r^2 values were 0.14 and 0.17, respectively (**Fig. 9E-F**). For NP and tamoxifen r^2 values were 0.46 and 0.58, respectively (**Fig. 9C-D**). For the steroidal estrogens EE_2 and E_1, r^2 values were 0.77 and 0.64, respectively (**Fig. 9A-B**).

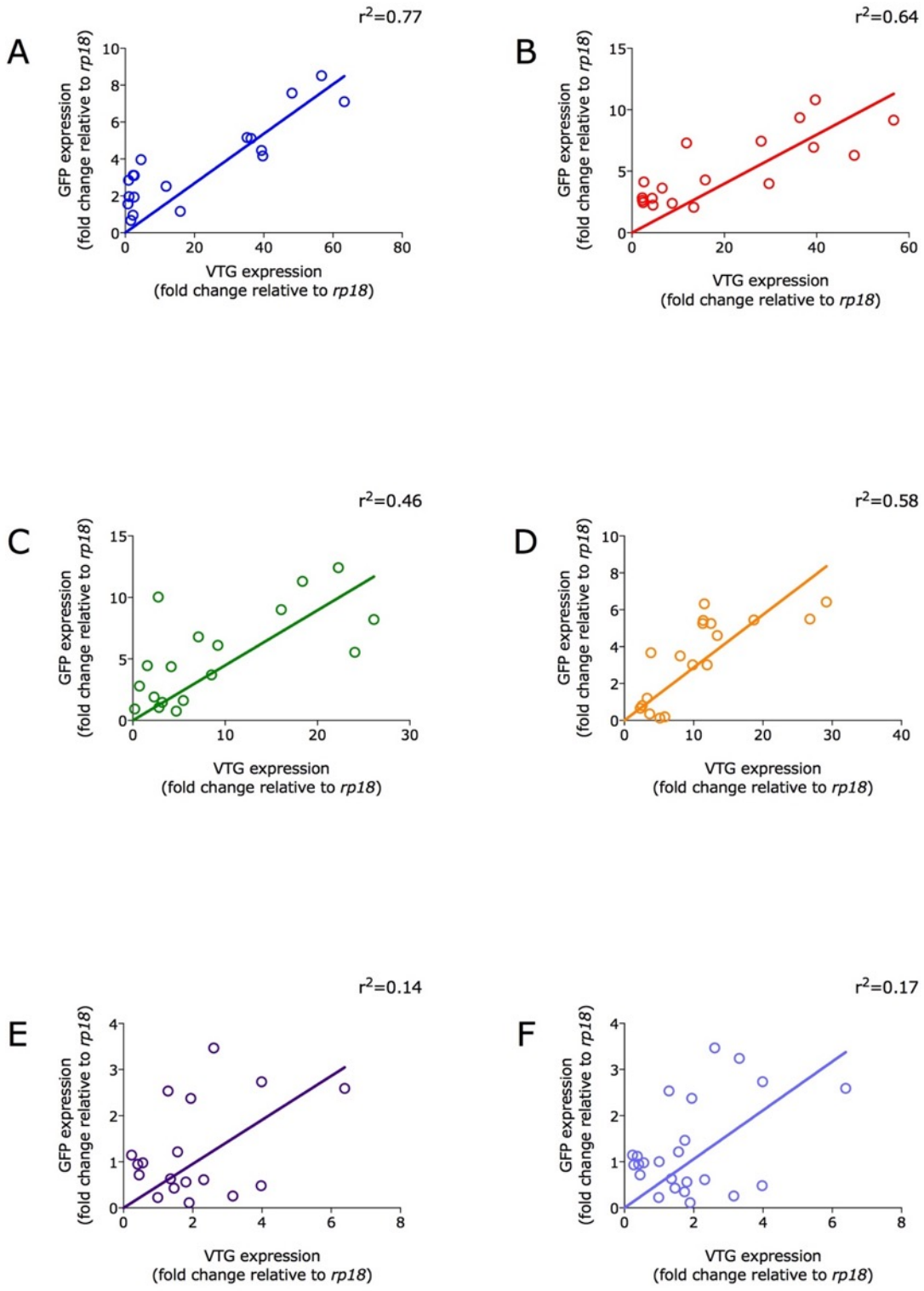

Figure 9. Correlation between GFP and VTG mRNA levels in ERE-GFP Casper zebrafish embryos following exposure from fertilisation to 96 hours post fertilisation to EE_2 (**A**), E_1 (**B**), NP (**C**), TAM (**D**), BS (**E**) and PFOA (**F**).

4.5 Discussion

Many chemicals found in the aquatic environment have been identified with oestrogenic properties and have been linked with reproductive and developmental effects with adverse health outcomes in fish. In this study we illustrate differences in the tissue expression of GFP for several suspected environmental estrogens in an estrogen responsive TG Casper zebrafish. We also show differences in tissues responses to given environmental estrogens for different early life developmental life stages. Unexpected responses for GFP expression include for both tamoxifen (a known estrogen receptor antagonist) and PFOA (a suspected estrogen receptor agonist). Finally, we also demonstrate further, the close relationship between *gfp* mRNA induction as measuring (capturing) estrogen respones across all the body tissues collectively and *vtg* mRNA induction occurring predominantly in the liver.

4.5.1 Biological responses in the ERE-GFP-Casper zebrafish model

4.5.1.1 Survival and hatching success

No treatment-related effects of EE_2, NP, tamoxifen or BS on embryo survival were observed and is agreement with that of other studies reported in the literature (Bugel *et al.,* 2016; Flinders *et al.,* 2014; Lei *et al.,* 2014; Versonnen and Janssen, 2004; Zha *et al.,* 2008). Survival was adversely affected for exposures to PFOA at the highest concentration (1000 µg PFOA $/L^{-1}$) and again in accordance with previous findings (Hagenaars *et al.,* 2011; Jantzen *et al.,* 2016; Zheng, *et al.,* 2012). PFOAs mediate toxicological effects through various mechanisms including via the activation of peroxisome proliferator activated receptor *γ* (PPAR*γ*) which can lead to alterations in the immune system (Grimaldi *et al.,* 2015). No signs of disease or infection that one might expect to see as a consequence of a suppressed immune system was observed in this study, and this therefore appears unlikely to be the mechanism accounting for the high mortality rate seen for this chemical. In addition, an increased mortality was observed in exposures to E_1 at the highest concentration (100 ng E_1 $/L^{-1}$) which has not been previously been found in the literature (to the best of this authors knowledge). This is important as E1 has been found to occur at high concentrations in surface waters but historically has been of lesser concern

because of its relatively lower affinity for ERs (Punnonen and Lukola, 1982; Ankley *et al.,* 2017).

A delay in hatching was seen in embryos exposed to EE_2 (10 ng EE_2 /L^{-1}) as has been reported previously in zebrafish and other fish species (Brazzola *et al.,* 2014; Versonnen and Janssen, 2004; Xu *et al.,* 2008). This was also the case for E_1 treated embryos at both 10 and 50 ng E_1/L^{-1}, but intriguingly not for the highest concentration (100 ng E_1/L^{-1}) and is an example of a non-monotonic dose response often reports in the steroidal oestrogens (Lagarde *et al.,* 2015). Reports in the literature of the effects of E_1 on this biological endpoint of are limited. Lei *et al.,* (2013) however, found that exposure to 50 ng E_1 /L^{-1}, significantly lowered the hatchability and lengthened the time to hatch of fertilized eggs in zebrafish. Due to its high lipophilicity (Liscio *et al.,* 2009) it would be expected that E_1 can transfer into the developing embryo from the water during the exposure time and thus interfere with the hatching process.

The observed delay in hatching seen in EE_2 and E_1 exposures may have resulted from an effect on proteolytic hatching enzymes, which are known to be required for chorion-softening and subsequent hatching in teleost fish (Kim *et al.,* 2006; Sano *et al.,* 2008). Possible disruptions in the regulation of the hatching gene tetraspanin cd63 has been suggested (Trikic *et al.,* 2011). Recently, a study by De la Paz *et al.,* (2017), found a strong inhibition of hatching in zebrafish embryos exposured to the fungicide triazole, a selective oestrogen receptor modulator (SERM). This was concurrent with a decease in the secretion of hatching gland cells, which have previously been shown to express all three ER subtypes during early zebrafish life stages (Chandrasekar *et al.,* 2010; Hao *et al.,* 2013).

4.5.1.2 GFP/VTG expression

<u>EE2</u>

Evidence concerning the oestrogenic effects of EE_2 is well established, with increases in *vtg* concentrations, feminization of fish, alterations to gonadal differentiation and reduced fecundity and/or fertility among the most commonly observed effects. (Muncke *et al.,* 2007; Silva *et al.,* 2012; Xu *et al.,* 2008; Yan *et al.,* 2012).

As with previous studies (Lee *et al.,* 2014, Green *et al.,* 2016; our own unpublished data), it was found that exposure to EE_2, induced GFP in a wide range of tissues, as expected given it is a highly potency as an ER agonist and a derivative of the natural hormone E_2. The binding affinity of EE_2 for the ER(s) has been reported to be up to five times higher than for E_2 in some fish species (Denny *et al.,* 2005; Shyu *et al.,* 2011).

It is perhaps no surprise that the LOEC for GFP induction in EE_2 exposed embryos (1 ng EE_2 /L^{-1}) was the lowest of all the tested chemicals, again confirming the high relative potency of this chemical and is within the range of levels commonly recorded in WwTW effluents (low ng/L^{-1}) and sometimes in surface waters (Adeel *et al.,* 2017).

Further analysis of *vtg* mRNA in the transgenic zebrafish found a significant upregulation of *vtg* expression, in all exposure groups, corroborating previous studies in the literature (Jin *et al.,* 2008; Muncke & Eggen, 2006; Sahoo & Oikari, 2013).

E1

Despite the fact that it is frequently detected in environmental samples[1], often at levels one to two orders of magnitude higher than that of EE_2 or E_2 (Alvarez *et al.,* 2013; Cavallin *et al.,* 2016; Chen *et al.,* 2010; Ma *et al.,* 2016) the potential ecological risks posed by E_1 have generally been viewed as less concerning, largely because it is less potent in terms of binding to and activating the ER(s) (Denny *et al.,* 2005; Peterson and Tollefsen, 2011; Lange *et al.,* 2012). This is despite evidence which shows a correlation between E_1 and the occurrence of feminization of fish in smallmouth bass (*Micropterus dolomieu*) (Blazer *et al.*, 2014) and other oestrogenic effects including *vtg* induction and alterations in secondary sex characteristics in fathead minnows (Dammann *et al.,* 2011). More recently too, a study by Ankley *et al.,* (2017), showed that exposure to environmentally relevant levels of waterborne E_1 resulted in plasma concentrations of E_2 in male fathead minnows similar to that of those found in

[1] Reported concentrations of E_1 in WwTW effluents typically range from 0.2–154 ng/L (Johnson et al., 2005; Williams et al., 2012).

reproductively active females, as a result of a conversion of E_1 to E_2 by one or more 17β-hydroxysteroid dehydrogenases. Hence, there is a need for a greater knowledge of the biological effects of E_1 in fish.

In this study, exposure to E_1 produced similar expression patterns of GFP, in terms of target tissue types, to that of EE_2, with additional GFP induction found in the pectoral fin. Several studies have shown that exposure to environmental oestrogens can inhibit breeding tubercle cluster formation in both intact and regenerating pectoral fins in male zebrafish (Pawlowski *et al.,* 2004; McMillan *et al.,* 2013). Here, the high sensitivity of the pectoral fin to E_1 would indicate that it plays a role in the development/function of fin systems in fish, but this would need to be investigated further to validate these results. E_1 has been found to have a high affinity for *esr2a* and *esr2b* (Cosnefroy *et al.,* 2012), which are expressed in a broad range of tissues including the neuromasts, pectoral fin buds, and liver (Celeghin *et al.,* 2011; Froehlicher *et al.,* 2009; Gorelick *et al*. 2014), and this is consistent with the GFP responses in our TG zebrafish for this chemical. The LOEC for GFP induction in E_1 exposed embryos (50 ng E_1 /L^{-1}) showed it to have a high relative potency, compared with the non-steroidal chemicals and is within the range of levels commonly recorded in WwTW effluents (low ng/L) (Adeel *et al.,* 2017).

Analysis of *vtg* mRNA expression in the ERE-GFP zebrafish found an induction of *vtg* for all E_1 exposures, corroborating the findings of previous studies by Ankley *et al.,* (2017) and Lei *et al.,* (2014). Thus, the results presented here add further evidence that E_1 may be more significant as an environmental oestrogen than has largely been reported. Additional studies, including for chronic and partial life cycle exposures, are therefore needed to achieve a deeper understanding of the potential for both environmental and human health effects of this environmental oestrogen.

Tamoxifen

In general, the oestrogenic effects of tamoxifen in fish are not widely reported but include reproductive effects such as reductions in fertility, fecundity and gonadal maturity (Mills *et al.,* 2016; Singh *et al.,* 2014; Sun *et al.,* 2007).

As with other human pharmaceuticals tamoxifen can enter the aquatic environment in its native form or as transformed excretory products, i.e. endoxifen and 4-hydroxy-tamoxifen, both of which demonstrate a higher affinity and specificity for the ERs than tamoxifen (Helland *et al.,* 2015). It has been detected in both hospital (Langford and Thomas, 2009) and municipal effluents (Zhou *et al.,* 2009) at levels reaching the high ng/L^{-1}, as well as in surface waters at concentrations reaching 212 ng/L^{-1} (Zhang *et al.,* 2013).

Unlike the other chemicals used in this study, tamoxifen is classified as a selective estrogen receptor modulator (SERM). SERMs are non-steroidal compounds which bind to ERα or ERβ subunits in the cell, interacting with EREs, leading to the activation of oestrogen-inducible genes (Komm & Mirkin, 2014). A unique feature of SERMs is their tissue-selective activity; once bound, they can elicit either agonistic or antagonistic activity dependent on the target tissue. This specificity is the result of several factors, largely tissue-specific expression of ER subtypes (ie, ERα and ERβ), differential expression of co-regulatory proteins in several tissues (ie, co-activators and co-repressors), and variable ER conformational changes generated by ligand binding (Martinkovich *et al.,* 2014 [**Fig 10**]).

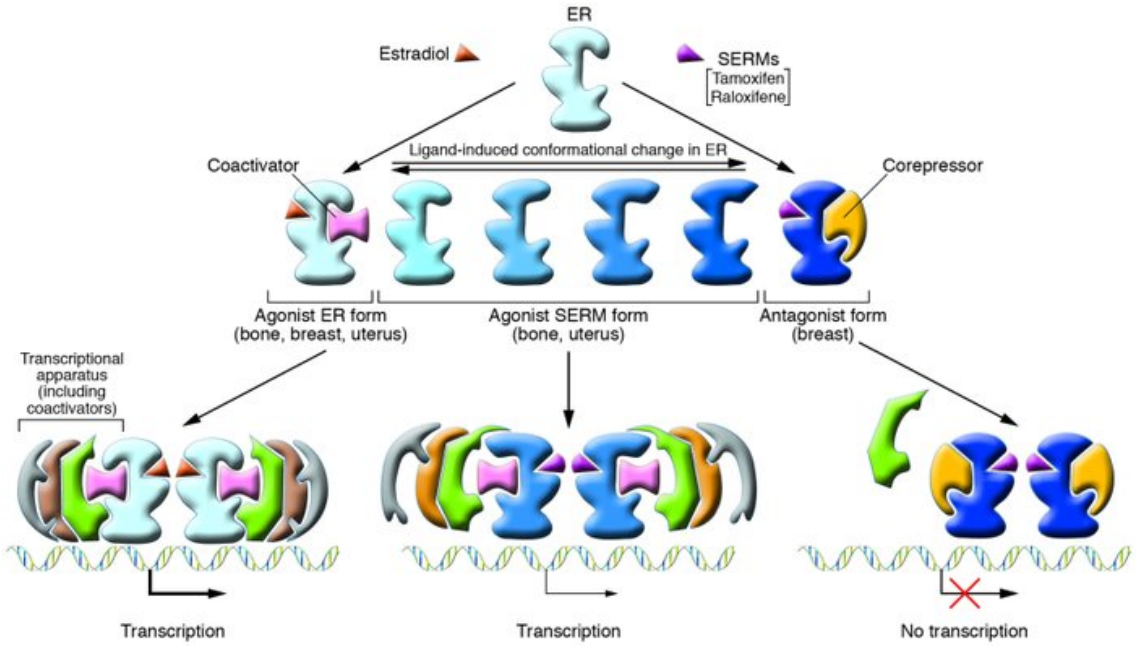

Fig 10. Differential ER structure and coactivator recruitment by ER agonists, antagonists, and SERMs, source Martinkovich *et al.,* 2014).

In mammalian pharmacology, on the basis of its tissue-specific behaviors, tamoxifen is an oestrogen agonist in the uterus, bone, and liver and a potent oestrogen antagonist in the breast, and thus is widely used to treat ER-positive breast cancer (Martinkovich *et al.,* 2014).

In this study, for embryo exposures to tamoxifen, GFP expression was similar to that of NP in terms of target tissues, with the strongest expression found in the liver, somites and cranial muscle. Previous findings have reported that exposure to tamoxifen can lead to increases in hepatic *vtg* levels in different fish species (Van der Ven *et al.* 2007; Vetillard & Bailhache 2006), whilst in humans, studies have also shown links with tamoxifen treatment and detrimental effects on the liver, including hyperplasia and increases in fatty liver tissue, albeit measured levels were significantly higher in these studies, i.e.mg/L (Hong *et al.,* 2017; Menescal *et al.,* 2012; Pan *et al.,* 2016). Thus the results from this study further substantiate tamoxifen as an oestrogen agonistic in the liver.

An unexpected finding from this study was the ability of tamoxifen to act as an agonist in the somites and cranial muscle. This is unusal given that it has previously been shown to act as an antagonist in tissues which express *esr2* such as the skeletal muscle (Velders *et al.,* 2012). For example, Patel *et al.,* (2017), found adverse reproductive effects in male mice exposed to 1mg tamoxifen/L^{-1} in drinking water including the impairment of spermatogenesis and steroid hormone production, which was linked to a decrease in *esr2* expression. Thus the results of this study add another level of complexity to the tissue-specific actions of this drug.

In comparison, tamoxifen has been shown to act as a partial agonist in tissues which express *esr1* (Barkhem *et al.,* 1998; Hall *et al.,* 1999). Previous research has shown that the heart displays a high level of expression of *esr1* (Chandrasekar *et al.,* 2010), which is further localised in different cardiac cell types (Gorelick *et al.,* 2011, 2014). Here, the ability of tamoxifen to induce GFP expression in the heart suggests that it is capable of activating *esr1*, and hence displays ER agonistic activity for this particular tissue.

The LOEC for GFP induction in tamoxifen exposed embryos (10 µg TAM /L^{-1}) is more than 10000-fold higher compared to the steroidal estrogens E_1 and E_2, and is higher than the range of levels generally recorded in WwTW effluents (low ng/L, [Zhou et al., 2009])

Further analysis of *vtg* mRNA in the transgenic zebrafish found a significant upregulation of *vtg* expression in 20 & 100 µg TAM /L^{-1}exposure groups, thus indicating an oestrogenic effect. This is in agreement with previous reports in the literature (Sun *et al.,* 2009, 2010) albeit these studies were conducted using adult life stages in both medaka and zebrafish, but with similar concentration ranges to those used in this study (e.g. 50 and 30 µg TAM /L_{-1} respectively).

Recently, a study by Xia *et al.,* (2016), found that exposure to tamoxifen resulted in a downregulation in *vtg* expression in zebrafish embryos, however the concentrations used were higher than those in this study (i.e. 500 µg TAM /L^{-1}). In mammalian pharmacology tamoxifen can also be considered as either an agonist or partial antagonist of the ER in different tissues according to different dose regimes. For example, Wang *et al.*, (2013) found a dose dependent effect of tamoxifen in breast cancer cells whereby low doses were found to act as an estrogen agonist via stimulation of the ERK1/2 signaling pathway, whilst high dose tamoxifen inhibited cell growth by blocking the activation of ERK1/2.

Overall, the results of the *vtg* analysis, combined with the ability of tamoxifen to induce GFP expression in a wide range of tissues in the ERE-GFP zebrafish model, suggests that tamoxifen might be considered more widely as an ER agonist, at least in terms of its effect on the early stages of teleost fish. The toxicological significance of a possible life-stage specific ER activity for tamoxifen warrants further investigation.

It is worth noting that tamoxifen has been shown to have a high affinity for the estrogen related receptor ERRγ (Coward *et al.,* 2001). ERRs are orphan nuclear receptors which are able to bind to functional EREs in ER target genes, producing translational responses (Giguere *et al.,* 1988). The tissue expression patterns of ERRγ are widely distributed, with high levels of expression seen in the heart, kidney, brain and placenta (Heard *et al.,* 2000). Thus, it is theoretically possible

that the GFP responses observed in the ERE-GFP zebrafish for tamoxifen resulted partially from ERR-induced promotion.

NP

The oestrogenic effects of alkylphenols such as NP in teleosts are well documented, both *in vivo* and *in vitro*, and includes induction of *vtg*, developmental effects, reproductive effects (e.g. reduction in egg production) and the occurrence of intersex (Ali *et al.,* 2011, 2017; Chandrasekar *et al.,* 2011; Shelley *et al.,* 2012; Won *et al.,* 2014).

In this study, GFP expression patterns in embryos exposed to NP, matched those previously seen in the oestrogen responsive TG models (Green *et al.,* 2016; Lee *et al.,* 2012), with strongest expression found in the liver, heart and somites. Whilst several studies in fish have shown how exposure to NP can impact on the growth and function of the liver, with effects including decreases in weight and hepatosomatic indices, liver necrosis, and fatty tissue infiltration (Abdulla, 2012; Midhila & Chitra, 2015), the cardiac effects of NP remain unclear and largely unexplored. Studies by Ali and Legler (2011), and Lee *et al.,* (2012), found exposure to NP resulted in irregularities in the heart rate of zebrafish and medaka embryos, from 48hpf, with no significant effects on heart development per se. The results seen here add further evidence for the potential of NP to target the heart, and this may be as a result of the known characteristic of NP to preferentially bind to *esr1* (Amaro *et al.,* 2014).

The LOEC for GFP induction in NP exposed embryos (20 µg NP /L^{-1}) is more than 10000-fold higher compared to the steroidal estrogens E_1 and E_2, and this effective concentration is within the range of levels recorded in WwTW effluents (low µg/L) (Soares *et al.,* 2008). NP has been shown to have an affinity for Erα that is about a 1000-fold lower than that of the natural ligand E_2 (Olsen *et al.,* 2005) and this may account for the higher LOEC observed here. It has also been reported that *in vivo*, NP can be extensively transformed in fish to less potent metabolites as oestrogens (Bone, 1995; Lewis and Lech, 1996).

BS

In embryos exposed to BS, tissue expression patterns were similar to that induced by the plasticizer bisphenol A (BPA) in previous studies with our TG zebrafish model (Lee *et al.,* 2012), although GFP induction was significantly weaker in the heart compared to BPA at the same concentrations. The induction of GFP expression in the heart has also been observed in an ERE-GFP-TG zebrafish model by Gorelick and Halpern (2011), specifically in the heart valves, and in previous studies with the ERE-GFP Casper zebrafish (Green *et al.,* 2016) after exposure to the phytoestrogen, genistein, which has a chemical structure comparable to that of BS.

BS has been shown to be a weak agonist for *esr1* and *esr2*, and preferentially binds to *esr2* (Gutendorf and Westendorf, 2001). As with tamoxifen, reports of the Oestrogenic effects of this chemical in the literature are limited, but some studies have reported that it can cause several endocrine effects in fish including modifications to gonadal steroidogenesis expression and alterations in steroid biosynthesis and lipid metabolism (Sharpe *et al.,* 2006, 2007). The induction of *vtg* has also been reported in some fish species (Nakari & Erkomaa, 2003; Orrego *et al.,* 2010). This is in direct contrast to observations found in this transgenic zebrafish model and is possibly reflective of both a longer exposure period and different methods of exposure, i.e. via intra-peritoneal injections, used in these studies.

In this study, the LOEC for GFP induction in BS exposed embryos was 100 µg BS $/L^{-1}$, thus showing a low relative potency of this chemical compared to the steroidal oestrogens E_1 and E_2 (100000 fold higher), and is considerably higher than that of levels commonly recorded in both surface waters and WwTW effluents (in the range of ng/L) (Liu *et al.,* 2010). However, when compared to the oestrogenic activities of other well documented environmental oestrogens such as NP and BPA, many phytoestrogens, including BS, are in the same order of magnitude in terms of differential binding to the ERs (Lin *et al.,* 2013; Liu *et al.,* 2009) and therefore any potential oestrogenic effects derived from BS should not be discounted particularly when they occur in higher concentrations in the environment, along with other oestrogenic phytoestrogens. In addition, given its similar response pattern, in terms of target tissues, to BPA, which has been

associated with heart disease, diabetes and obesity (Bertoli *et al.,* 2015; Han and Hong, 2016), the potential for human health risks should also not be ruled out.

PFOA

Several studies in the literature have indicated the potential of the perfluorinated compound PFOA to induce oestrogenic effects in fish. For example, exposure to PFOA has been reported to increased *vtg* expression in cultured tilapia hepatocytes (Liu *et al.,* 2007) from rare minnows (*Gobiocypris rarus*) (Wei *et al.,* 2008) and rainbow trout (Benninghoff *et al.,* 2011). A study by Tilton *et al.* (2008), found dietary exposure to PFOA induced an overall hepatic gene expression profile similar to that of E_2 in rainbow trout. A study by Du *et al.* (2013), demonstrated that exposure to PFOA were capable of increasing levels of *esr1* (but not *esr2b*) and the steroidogenic enzyme *cyp19b* in zebrafish embryos, suggesting that in these instances, the oestrogenic effects of PFOA were mediated through the ER.

In all of these studies the concentrations of PFOA required to initiate these responses were in the µg/L to mg/L range, showing it to have a far weaker oestrogenic potency compared with many (most) other environmental oestrogens. In this study, PFOA did not induce any visible GFP expression in the different target tissues, or other measurable GFP responses as quantified by both Western blot and mRNA analysis. The lack of a detectable oestrogenic response may relate to the low bioavailability of the chemical to the exposed fish. Analytical analysis was not conducted on PFOA and given its hyrophobic nature it is highly likely that the amount of PFOA our fish were actually exposed to may have been much lower because of its tendency to stick to the glass dishes, therefore the concentrations shown are nominal, and not necessarily the actual concentrations our fish experienced. Another explanation for the lack of a detectable oestrogenic response in the ERE-GFP-Casper model is that PFOA may not exert oestrogenic effects through the classical (genomic) pathway (Bjornstrom and Sjoberg, 2005) and thus is not captured in this model.

Several studies have also shown that ERs can regulate the expression of a large number of oestrogen-responsive genes whose promoters do not contain EREs. This is achieved without binding directly to DNA, through protein-protein

interactions with other transcription factors such as Fos/Jun (AP-1-responsive elements) or SP-1 (GC-rich SP-1 motifs) after ligand activation (Kusher *et al.,* 2000; Saville *et al.,* 2000). In addition, other rapid 'non-genomic' and ligand-independent pathways have also been described by which oestrogens and ERs can regulate biological processes (Kato *et al.,* 1995; Simoncini *et al.,* 2004).

As for tamoxifen, PFOA has been also shown to exhibit antagonistic as well as agonistic properties for the ER. Henry and Fair (2011), found that PFOA exhibited oestrogenic as well as anti-oestrogenic activities using an E-screen assay. Hepatic expression of *vtg* has been shown to be closely coupled to E_2-dependent up-regulation of *esr1* expression in fish, with levels increasing simultaneously after exposure to oestrogenic chemicals (Adedeji *et al.,* 2012; Andreassen *et al.,* 2005; Zhang and Hu, 2008). In this study, it was found that exposure to PFOA had no significant effect on *vtg* induction, further supporting a possible antagonist activity of the ER for this chemical.

In terms of its potential for human and environmental health risks, whilst average concentrations of PFOA found in surface waters are fairly low, ranging from 0.2–1630.2 ng/L^{-1} (Kunacheva *et al.,* 2012), both the chemical and physical properties of PFOA (i.e. its hydrophobic/lipophobic character and thermal and chemical stability in extreme conditions) mean that it can bio-accumulate in fish, (Hong *et al.,* 2014**)** with recently reported levels of 125 ng/g found in whole body zebrafish, after exposure for 5 days to 10 ug/L PFOA (Ulhaq *et al.,* 2015). Reports of PFOA in humans are also common, where levels have been recorded in the ng/mL, in human blood (Kannan *et al.,* 2014).

Overall, analysis of GFP expression in the ERE-GFP Casper zebrafish showed that higher exposure concentrations for the tested chemicals gave rise to progressively higher GFP fluorescence (with the exception of PFOA) as confirmed quantitatively via Western blot analyses. As this model is designed to detect responses to all ER subtypes it cannot be infered from the results seen here whether the different chemicals tested are facilitating GFP expression via the same or different ERs (or potentially ERRs). The use of morpholinos for antagonising specific ER subtypes, or CRISPR-Cas or TALENS (used to silence ER subtype expression) in the Casper model could aid in the clarification of the exact mechanisms of action for different environmental oestrogens.

As with previous work (our own unpublished data) direct comparison of GFP with VTG mRNA expression demonstrated a greater sensitivity for the latter, at least in terms of the magnitude of responses. This might be expected considering the huge induction capacity for VTG (and its protein product) of up to a million-fold (Tyler *et al.,* 1996) and may reflect differences in the dynamics of synthesis and turnover in mRNA between the two genes. However, there is more than one *vtg* mRNA, with at least 7 known sub-types in zebrafish (Meng *et al.,* 2010) and these mRNAs may have different responsiveness to estrogens. A greater scope for the magnitude of change for *vtg* also means that it is more likely to see changes/induction with a statistical significance compared to GFP induction which is much more variable.

4.6 Conclusion

This study found tissue-specific effects in response to the different oestrogenic chemicals in the ERE-GFP-Casper zebrafish model, further substantiating its high sensitivity and applicability for use in environmentally relevant toxicological research and chemical testing and screening. Recently this ERE-GFP-Casper transgenic model has been developed further with a Kaede photoconvertable (green to red) fluorescent protein, to create an ERE-Kaede-Casper zebrafish that will potentially allow for effective studies into temporal dynamics and mixture responses to oestrogens (Green *et al.,* 2018). Differences in the tissue specificities seen in this study are likely to have occurred as a result of differences in tissue expression and interactions with the three ER subtypes (*ers1, ers2a, ers2b*) and other related receptors that can form heterodimers with ERs.

Although preliminary in nature, some of the findings using the ERE-GFP Casper zebrafish question current understanding regarding modes of action for certain estrogenic chemicals, i.e. tamoxifen and PFOA, and intimate at possible novel life-stage specific agonistic behaviour, although the latter is purely speculative at this time.

Future investigations using the transgenic model system could help to further identify and characterise tissue specificities for other oestrogenic chemicals of widespread concern, in particular, selective oestrogen receptor modulators (SERMs). Earlier identification of the intrinsic properties and modes of action of

suspected oestrogenic chemicals using the ERE-GFP Casper zebrafish model, may be a step forward in realising how to accurately assess the risks posed by these chemical pollutants, both in the aquatic environment, and on human health.

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4.9 Supplementary Information

S1. Chemical structures of the selected oestrogenic compounds .

S2. Generation of ERE-GFP-Casper (F0) line. TG1 indicates ERE-Gal4ff transgene sequence. TG2 indicates UAS-GFP transgene sequence. Expression of pigmentation genes *roy* (dark) and *nacre* (silver) are also shown. The ERE-GFP model, homozygous for both transgenes, and a Casper strain were initially crossed to give a heterozygous generation. Larvae were then screened for the homozygous ERE/Casper genotype and individuals carrying both the Casper phenotype and GFP expression were raised to sexual maturity. Fish were then screened for homozygous expression of the transgene sequences to produce the founder generation (F0) of the ERE-GFP-Casper line.

S3. Protocol description of LC-MS used for chemical detection of the environmental oestrogens in the water.

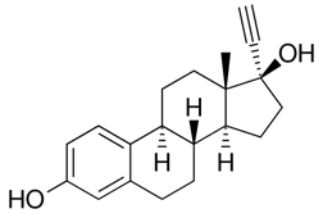
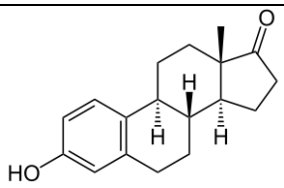
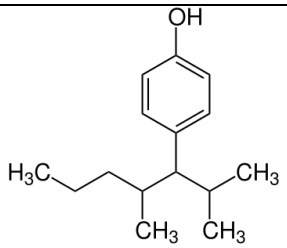
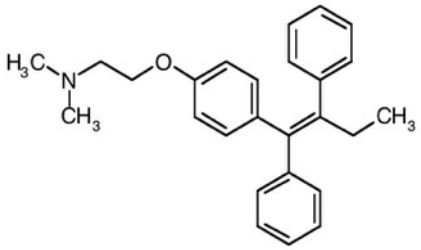
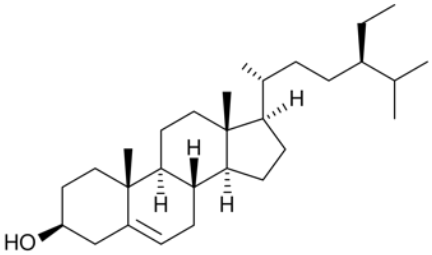
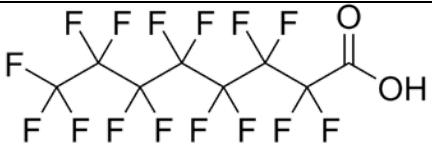
Chemical	
EE2	
E1	
NP	
Tamoxifen	
BS	
PFOA	

Table S1.

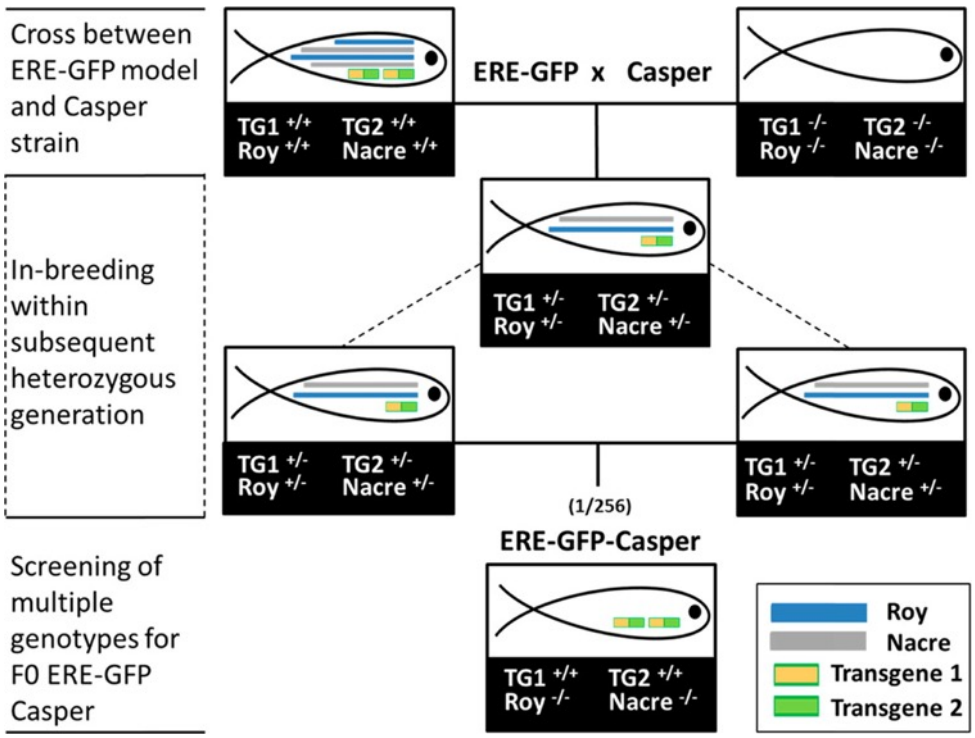

Cross between ERE-GFP model and Casper strain
In-breeding within subsequent heterozygous generation
Screening of multiple genotypes for F0 ERE-GFP Casper
ERE-GFP x Casper
TG1 +/+ Roy +/+
TG2 +/+ Nacre +/+
TG1 -/- Roy -/-
TG2 -/- Nacre -/-
TG1 +/- Roy +/-
TG2 +/- Nacre +/-
(1/256)
ERE-GFP-Casper
Roy
Nacre
Transgene 1
Transgene 2

Figure S2.

S3. Water Chemistry LC-MS Quantification

Analyses of water samples for exposure were performed using a Surveyor MS Pump plus a HPLC pump with an HTC PAL autosampler coupled to a TSQ Vantage triple quadrupole mass spectrometer. The mass spectrometer was equipped with a heated electrospray (HESI II) source (all ThermoFisher Scientific, Hemel Hempstead, UK). Chromatographic separation was achieved using a reversed-phase, 3 µm particle size, C18 Hypersil GOLD column (50 mm × 2.1 mm i.d., Thermo Scientific, San Jose CA, USA).

Analytes were separated using a linear gradient of (A) aqueous phase and (B) organic solvent containing additives specific for analysed compounds. Solvent B increased to 100% in for all analytes. This was maintained for 1.5 min for before returning to the initial condition. The flow rate was 500µL/min. Temperature of autosampler was set at 8˚C while column was kept at a room temperature.
The HESI probe was operating in both negative and positive mode; an ion-spray voltage of -2.75 kV was applied for analysis of BS, +3.75 kV and for 4.0 kV for remaining compounds. The heated capillary temperature was set at 275 °C and the vaporizer temperature was 60 °C for BPA, 500°C for nonylphenol and 350°C for remaining compounds. Nitrogen was employed as a sheath and auxiliary gas at a pressure of 60 and 2 arbitraryunits, respectively.

The argon CID gas was used at a pressure of 1.5 mTorr and the optimum collision energy (CE) for each transition was selected. Quantification of the target compounds was performed by monitoring two characteristic multiple reaction monitoring (MRM) transitions.

Chapter 5

Investigating expression of endogenous GFP in ERE-GFP zebrafish as a possible way to sex fish during early life

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5.1 Abstract

Teleost fish exhibit a wide variety of sex determination mechanisms, which range from systems under strict genetic control to those that include both genetic and environmental factors, such as temperature. The zebrafish (*Danio rerio*), has many advantages as a model system, but to date, mechanisms controlling its sex differentiation and determination remain elusive, which is seen as a limitation for this species as a model organism.

In this study, an oestrogen element responsive (ERE), green fluorescent protein (GFP), Casper transgenic zebrafish was used to determine if endogenous GFP expression during early life could be used as a sex marker in this model. The ontogeny of endogenous GFP expression in individual fish was followed at 10-day intervals, from early life stages (5 days post fertilisation), through the period of sexual differentiation using fluorescent microscopy. At 60 days post fertilisation (dpf) fish were sacrificed and their phenotypic sex determined using histological analysis.

Levels of endogenous GFP expression varied across the different zebrafish life stages and this is likely a reflection of the varying functional roles they perform during development. Expression of GFP in the different tissues in individual fish was not detectable after 30 dpf, most likely due to fluorescence quenching due to the skin thickness. During early life there appeared to be four main groups of fish identified based on both the timing and duration of tissue GFP expression. In group A zebrafish, no GFP was observed (9% of total population), in group B, GFP expression was seen in the heart from 5 to 10 dpf (33% of fish), in group C, GFP expression was seen in the otic vesicle in 34% of the population, whilst in group D, GFP was expressed in the liver only at 30 dpf up until 40 dpf. Gonadal sex however, was linked to the pattern (intensity) of GFP expression in group D only (where enhanced expression was linked to the female phenotype at 60 dpf). This life period coincides with the timing of sexual differentiation of the gonad in zebrafish. Thus, these results show that endogenous GFP expression cannot be used to distinguish gonadal sex in this transgenic zebrafish line prior to gonadal differentiation using fluorescence microscope but can however be utilised as a phenotypic marker for non-destructively identifying females at 30 dpf.

5.2 Introduction

The zebrafish (*Danio rerio*) has become an important experimental model organism due to its well-known conveniences for genetic manipulations and genome-wide analyses. It is used extensively, and increasingly so, as a model to inform on developmental and disease processes in humans and has also gained rapid recognition in the screening and testing of chemicals to identify potential chemical hazards for risk assessment.

Despite this popularity, very little is known about sex determination (SD) mechanisms in zebrafish, with much of the literature pointing towards a polygenic sex determination system, where the sex of an individual is determined by different allelic combinations of loci and not by the absence of presence of a specific chromosome (Bradley *et al.,* 2011; Anderson *et al.,* 2012; Liew *et al.,* 2012; Howe *et al.* 2013). Several studies have also shown that SD in zebrafish can be influenced by environmental factors such as temperature, hypoxia, population density and food availability (Shang *et al.,* 2006; Lawrence *et al.,* 2008; Abozaid *et al.,* 2011; Sfakianakis *et al.,* 2012; Villamizar *et al.,* 2012; Luzio *et al.,* 2016). Moreover, SD mechanisms have also been shown to vary among different zebrafish strains (Anderson *et al.,* 2012; Wilson *et al.,* 2014).

Historically, zebrafish have been classed as an undifferentiated gonochoristic species (Yamamoto, 1969), where, prior to sexual differentiation and irrespective of genetic background, all individuals develop an ovary-like gonad (termed a “juvenile” ovary). This ovarian development can be observed from as early as 3 weeks post fertilization (wpf) to around 4 wpf (Takahashi, 1977; Maack and Segner, 2003). Subsequently, the gonad either continues to mature into a functional ovary, or testis development is initiated, where apoptosis of the ovarian cells and the ovarian cavity occurs (review; Orban *et al.,* 2009). However, several reports in the literature have shown that not all zebrafish males develop a juvenile ovary, presenting evidence for the possible existence of two pathway processes of gonad development in male juvenile zebrafish (Wang *et al.,* 2007; Luzio *et al.,* 2015, 2016).

Sexual differentiation in zebrafish is known to be regulated by the sex steroids, 17β-estradiol (E_2) and 11-ketotestosterone (11KT), which are responsible for the

induction and maintenance of ovarian and testicular development respectively (Nakamura *et al.,* 1998; Baroiller *et al.,* 1999). The balance between oestrogens and androgens (as catalysed by the cytochrome P450aromatase complex), has been shown to be critical in gonadal sexual differentiation, in such a way that excess E2 induces feminine differentiation, while excess 11KT induces masculine differentiation (Fenske and Segner 2004; Guerrero-Estévez and Moreno-Mendoza, 2010). The actions of these steroids are in turn mediated through the three oestrogen receptor (ER) forms found in fish (*ESR1*, *ESR2b* and *ESR2a*) (Bardet et al., 2002), and the androgen receptor (*AR*). In particular, the differential expression of the ERs has been suggested as essential in the early stages of sexual differentiation in zebrafish (Menuet *et al.* 2002).

Unravelling the mechanisms of SD in zebrafish is further complicated by the difficulties with differentiating between male and female zebrafish, both morphologically and genetically, before sexual maturation. Furthermore, attempts to identify expression of sex-dimorphic genes related to the gonad differentiation process in zebrafish, have all been hindered due to an inability to determine the individual sex of fish during the sex determining period (Hill and Janz, 2003; Sreenivasan *et al.,* 2008).

Currently, techniques for the determination of phenotypic sex in zebrafish are largely restricted to histological examination of the gonads, which is a destructive process. Sex-linked genetic markers offer a non-lethal method for the identification of genotypic sex and have been successfully developed in several species of teleost fish with a heteromorphic (XY) sex chromosome-based SD system. These include the only sex-determining gene found among the non-mammalian vertebrates (to date), *DMY* (*DM*-domain gene on the *Y* chromosome), discovered in the medaka (*Oryzias latipes*), by Matsuda *et al.,* (2002). The presence of a heteromorphic XY pair corresponding to linkage groups (LG) 19 and 12, has also been found in the three spine stickleback (*Gasterosteus aculeatus*) and the nine spine stickleback (*Pungitius pungitius*) respectively, whilst in black-spotted stickleback (*G. wheatlandi*) males, one copy of LG12 has fused to the LG19-derived Y chromosome, giving rise to an X_1X_2Y SD system (Ross *et al.,* 2008, 2009; Shapiro *et al.,* 2009). In zebrafish, *DMY* has been found to be necessary for male sexual development (Jorgonson *et*

al., 2008; Webster *et al.,* 2017) and is also expressed in the developing germ cells of the ovary, suggesting that it may also be important in ovary differentiation (Guo *et al.,* 2005). However, the identity of a sex determining gene in the zebrafish remains elusive.

The ability to correctly differentiate between the sexes is essential for studies where sexual difference needs to be accounted for. For example, appropriate interpretation of biological effect measures, i.e. the induction of vitellogenin (*vtg*) in response to exposure to environmental oestrogens, is only possible when individual sex is known (Holbech *et al.* 2006). Studies using transgenic fish lines expressing *vasa-gfp* (Krovel and Olsen, 2004; Presslauer *et al.,* 2016), *zpc-gfp* (Onichtchouk *et al.,* 2003), and *actin-gfp* (Hsiao and Tsai, 2003; Akhter *et al.,* 2016) have identified sexual dimorphic GFP expression in the gonads of zebrafish, which may be used to distinguish between males and females. However, these dimorphic expression patterns are limited to early juveniles (around 21 days post hatching or above), i.e. post the initial phase of the sex-determining period.

Previous investigations using an ERE-TG zebrafish, developed at the University of Exeter, have identified GFP expression occurring in early life stages (4 dpf) at low levels as a natural response (i.e. without exogenous oestrogen exposure), both in the otic vesicle and also in the heart (Lee *et al.,* 2012), and the suggestion is that these differences may relate to the presence of endogenous oestrogen during these early developmental stages. Endogenously induced expression of GFP has also been observed in other tissues, predominately in the liver, (and in the brain), at around 30 dpf. Anecdotally, this endogenously induced expression of GFP in the ERE-TG zebrafish has been reported to occur in around half of the fish population, suggesting it may relate to the sex of the individuals, as females will contain higher levels of endogenous oestrogen even during early life stages compared with males. Thus, the hypothesis is that fish expressing GFP during early life in populations not subjected to exogenous oestrogen treatment are females.

The aim of this study was to set out to undertake a simple experiment to attempt to determine whether natural endogenous GFP expression in the ERE-TG Zebrafish was sex related and if this could then function as an early sex marker, thus allowing

the identification of putative male and females, before/during the sex-determining period. The ability to identify individual sex in zebrafish (particularly in earlier life stages) would allow for a more comprehensive assessment of the health effects of endocrine disrupting chemicals (EDCs) and could also help to form a better understanding of the evolutionary trends in sex-determining systems.

5.3 Materials and methods

5.3.1 Test Animals and Husbandry.

A transgenic zebrafish strain developed at the University of Exeter, ERE-GFP Casper (3×ERE:Gal4ff and UAS:GFP) was employed in this work. The ERE-GFP Casper line was derived from crossing an established transgenic ERE-GFP line (Lee *et al.,* 2012) with a Casper strain acquired from University College London (**SI 1)**. This line contains silenced *roy* (dark) and *nacre* (silver) pigmentation genes, resulting in highly translucent skin (White *et al.,* 2008).

All zebrafish were raised at 28.5+/-1 ^{0}C under a 12-h light, 12-h dark cycle photoperiod regime, fed to satiation three times daily with ZM Fry food 000/100 (Zebrafish Management Ltd., Wincester, U.K.) and Liquifry No. 1 (Interpet, Dorking, U.K.) up to 21 dpf, progressing to live newly hatched Brine Shrimp (*Artemia*) and frozen Gamma Omega 3 Enriched Brineshrimp (supplied by Zebrafish Management Ltd., Wincester, UK) from 21dpf to 60 dpf.

5.3.2 Water supply.

The supply of water to the laboratory dosing system was maintained using reverse-osmosis with the addition of salts (as described in OECD Guideline 203). Water temperature and pH levels were monitored daily and ranged between 26.5 and 27.6 ◦C, with pH levels between 6.8 and 7.7. Dissolved oxygen concentrations were maintained at >80% of the air saturation value throughout all exposures. Conductivity ranged from 200 to 260 µS/cm.

5.3.3 Experimental Design.

ERE-GFP Casper zebra fish were randomly separated into 5 groups (*n* = 40 per group) from 24hpf and screened, using fluorescence microscopy, for endogenous GFP expression at the following time points: 5, 10, 20, 30, 40, and 50 dpf (**Fig. 1**). All fish were examined under fluorescence microscopy at 5 dpf and separated based on those, exhibiting fluorescence and those that did not (including in the otic vesicle and or/heart), and subsequently placed into individual holding tanks. From 10 to 50 dpf, all fish were examined every 10 days via fluorescence microscopy as they progressed throughout development to determine the dynamics of the GFP

expression. It was investigated whether those fish expressing GFP at 5 dpf continued to do so (or not), and whether other individual fish not expressing GFP at 5 dpf started to express GFP at later stages of development. Thus, in this experimental design, the ontogeny of any fluorescent tissues in each individual could be followed in real time. All fish were terminated at 60 dpf and gonadal histopathology was conducted to identify their gonadal sex. In addition, a sub population of 40 fish were exposed to 10 ng EE_2/L, as a positive control.

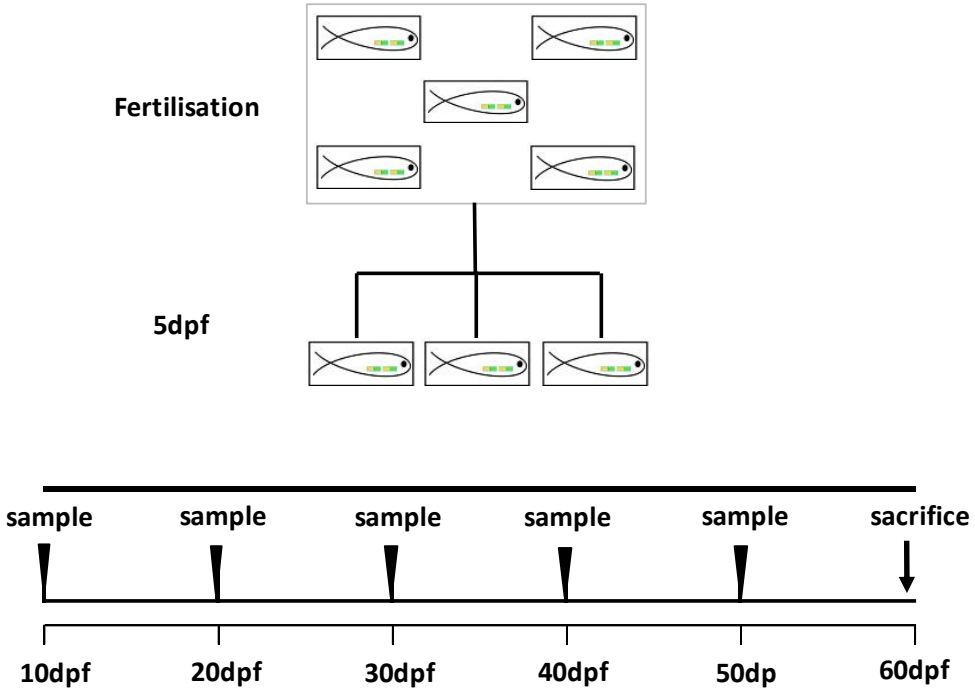

Figure 1. Schematic diagram showing the experimental design to measure the onset and duration of fluorescent tissue among individual zebrafish. Arrowheads indicate sampling days (5, 10, 20, 30, 40, 50 dpf), where all fish were examined via fluorescence microscopy to determine the dynamics of GFP expression as they progressed throughout development, and the arrow shows when fish were sacrificed (60 dpf).

5.3.4 Fish sampling and analysis of phenotypic endpoints.

All animal use protocols were carried out ethically in accordance with U.K. Home Office guidelines (Animals (Scientific Procedures) Act 1986). Fish were sacrificed by terminal anesthesia with benzocaine (0.5 g/L; ethyl-*p*-aminobenzoate; Sigma, Poole, U.K.). Male and female body weight, length and condition factor were measured at

60dpf. The condition factor (*K*-factor) was calculated for each fish using the formula: *K*-factor = (weight (g)/length $(cm)^3$) × 100.

5.3.5 Image analysis.

Fluorescence microscopy (Zeiss Axio Observer.Z1; Zeiss, Cambridge, UK) was used to visualise GFP expression in our zebrafish employing the Axiovision digital software programme. GFP expression was measured at 5, 10, 20, 30, 40, and 50 dpf respectively. Prior to live imaging all fish were anesthetized with 0.4% tricaine, mounted in 4% methylcellulose in a glass-bottom dish and oriented in dorsal, ventral and lateral views. All final images were prepared using Adobe Photoshop CS4 software (Adobe Systems, San Jose, CA, USA) to adjust light and contrast, as required.

5.3.6 Histological analysis.

All samples were fixed *in toto* in Bouin's fixative (Fisher Scientific) for a maximum of 6 hours and washed twice in 70% ethanol (at 12 and 24 hours post sampling) and subsequently processed for 16 hours in a semi-enclosed tissue processor (Shandon Citadel 2000). In brief, tissue samples were progressively dehydrated through a series of graded industrial methylated spirits (IMS) solutions (70-100%), washed in ethanol and Histoclear and then embedded in paraffin wax at 60 ^{o}C. Each zebrafish was then serially sectioned into 5-µm sections on a rotary microtome (Leica model). For each fish, a minimum of 6 transverse serial sections were obtained (taken at 50-µm and 250-µm intervals along the gonad for males and females respectively) mounted onto glass slides and subsequently stained with hematoxylin- eosin, before examination using a Zeiss Axioshop 40 light microscope (magnification 10-100x) to determine for gonadal sex. Gonadal sexes were confirmed based on the standardised criteria as defined in the OECD Histopathology Guidance document (Johnson *et al.,* OECD, 2009) and using a numerical staging system (see SI 2).

5.3.7 Statistical Analysis.

Data was checked for normality using the D'Agostino-Pearson test and for homogeneity of variance using Levene's test prior to statistical analysis. Data meeting these tests were analysed using parametric tests, e.g. analysis of variance (ANOVA) procedures. Any significant differences from expected sex ratios or stages

of gonadal development were tested using the chi-square test. All statistical analyses were run in GraphPad Prism 7 (GraphPad Software, Inc., San Diego, USA). Statistical significance was accepted as $p < 0.05$ for all comparisons and values are quoted as mean values and ranges as standard error of the mean.

5.4 Results

Accurate quantification of GFP expression via fluorescence microscopy was not possible in live fish over 30 dpf due to quenching of the fluorescence signal because of skin thickness. Thus, GFP data are reported in ERE-GFP Casper zebrafish are for observations based on life stages from 5 dpf to 30 dpf.

5.4.1 Body size/condition factor

Analysis of the weight and length in ERE-GFP Casper fish at 60 dpf showed that females were both significantly heavier and longer than males (p =<0.01 and <0.001, respectively; **Fig. 2A-B**). No significant differences in *K*-factor was found between sexes (data not shown).

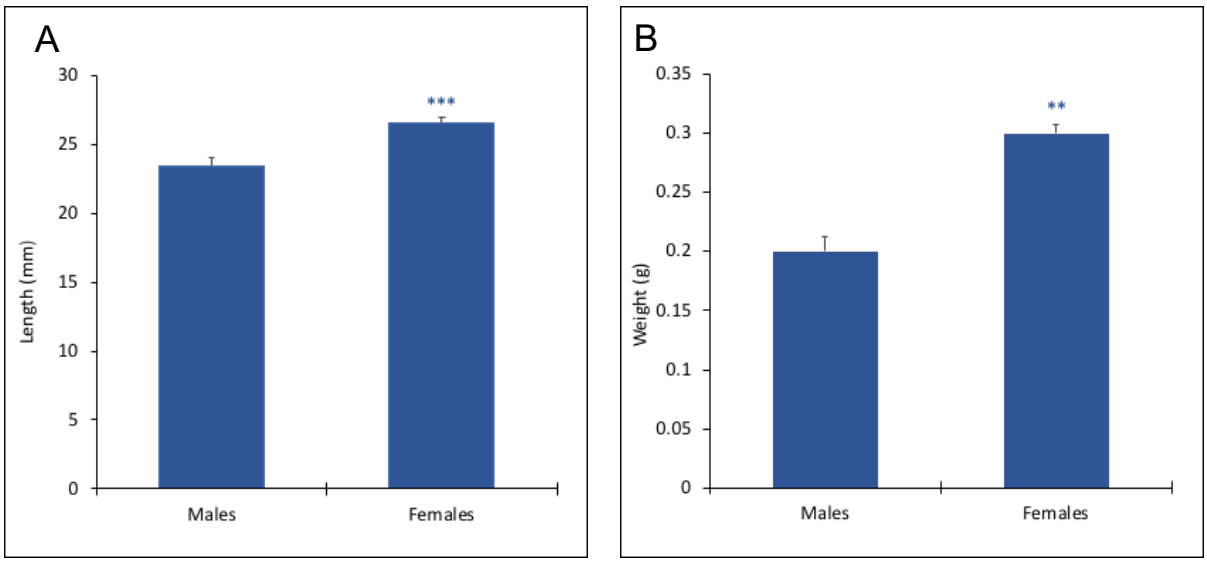

Figure 2. Length and weight as determined in male and female ERE-TG Casper zebrafish at 60 dpf. Data is reported as mean ± SEM. Asterisks denote a significant difference (p<0.01, ***p<0.001) males compared with females.

5.4.2 GFP expression

Different tissue responses of endogenous GFP expression were observed in our ERE-GFP Casper zebrafish at each sample point. At 5 dpf, GFP expression was seen in the heart and in the otic vesicle (**Fig. 3Bi**). In 10 dpf and 20 dpf larvae, GFP expression was only observed in the otic vesicle (**Fig. 3Bii-iii**). At 30 dpf, GFP expression was observed in the liver only (**Fig. 3Biv**).

In 10 ng EE_2/L exposed positive controls, GFP expression in 5 dpf zebrafish was observed in the heart (h), liver (li), somites (sm), forebrain (fb) and in the cardiac muscle (cm) (**Fig. 3Ci**) with comparable findings at 10, 20 and 30 dpf (**Fig.3Cii-iv**).

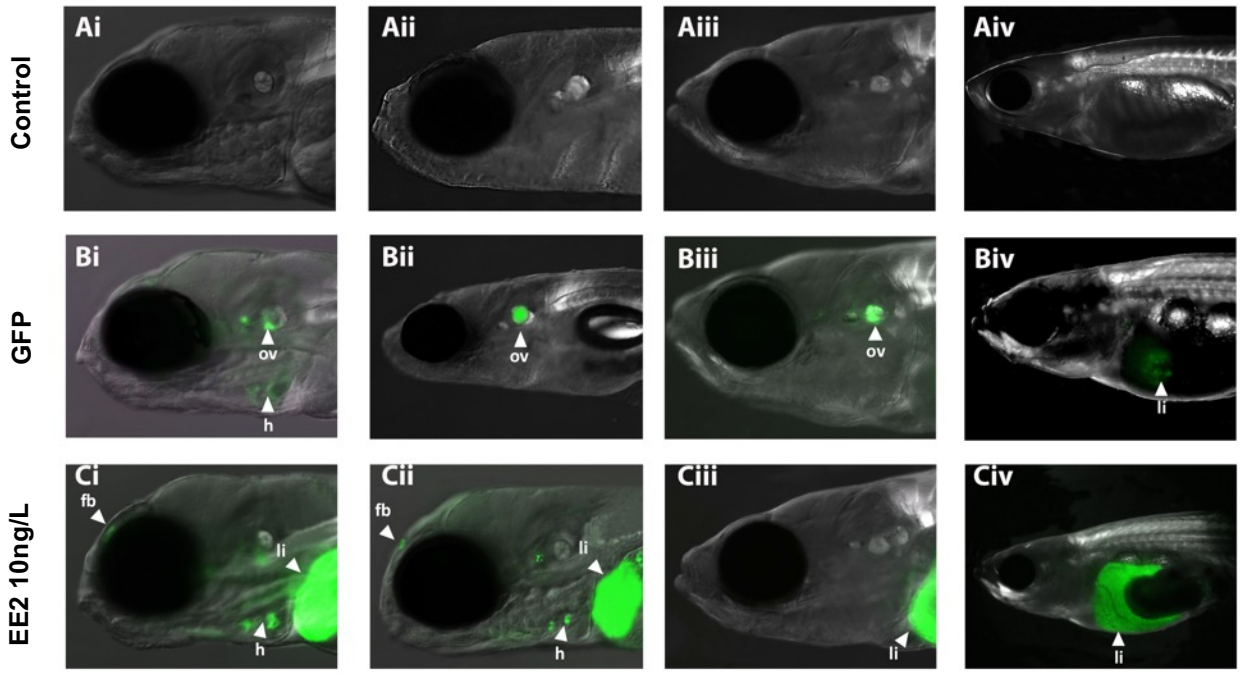

Figure 3. GFP expression in ERE-GFP Casper zebrafish. Data shows control fish (A), endogenous GFP expression in unexposed zebrafish (B) and GFP expression in zebrafish exposed to 10 ng EE_2/L, as a positive control (C), at 5dpf (i) 10dpf (ii) 20dpf (iii) and 30dpf (iv). Data is shown in the head with lateral views. GFP expression was observed in the heart (h), liver (li), and in the otic vesicle (ov). Bar = 200 μm.

Analysis of the ontogeny of expression of GFP in the different tissues in individual fish revealed that there were four main groups, based on both the timing and duration of tissue fluorescence (results are summarised in **Fig. 4**).

In Group A, no endogenous GFP was observed at any of the life stages studies, a total of 9% of the population (16/178 of individuals). Of these, 88% (14/16) were confirmed as males at 60 days.

In Group B, GFP expression was seen in the heart from 5 dpf up until 10 dpf in 33% (58/178) of the population. Of these, 33**%** (19/58) were female, and 67% (39/58)

male at 60 days. Within this subset of fish, of those individuals identified as female, 79% (15/19) also demonstrated GFP expression in the liver at 30 dpf.

In Group C around 34% (62/178) of the fish showed fluorescence in the otic vesicle at 5 dpf, which decreased to 28% (50/178) at 10 dpf, and 12% (22/178) at 20 dpf. Of these, 8**%** (5/62) of individuals were female, and 92% (57/62) were male at 60 dpf. In addition, in this group (C) of those individuals identified as female, 80% (4/5) also demonstrated GFP expression in the liver at 30 dpf.

In Group D, GFP expression was observed only in the liver at 30 dpf, up until 40 dpf, after which time the fluorescence signal was lost. Approximately 24% (42/178) of the entire population were in this group and after gonadal differentiation (at 60 dpf) all of these fish were confirmed as females via gonadal histopathology.

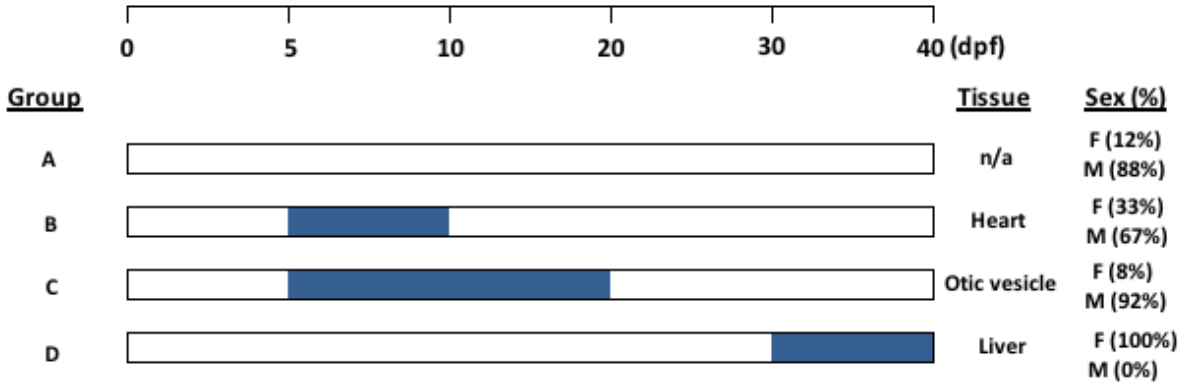

Figure 4. The four distinct groups of ERE-TG Casper zebrafish identified, based on where (tissue type) and when (stage of development) they displayed endogenous GFP expression. Filled blue boxes indicate the time of appearance of GFP expression and the duration. Unshaded boxes indicate no GFP expression. Percentages of males and females found are given for each group.

5.4.3 Sex ratio, sexual differentiation and development

There was a sex ratio bias in favour of males in the population of around 55:34 male to female ratio, which is normal for this species and in zebrafish strains (**Fig. 5A**). Of those fish sampled at 60 dpf and found to be female, 54% were classified as having Stage 3 ovaries, with the majority of developing follicles consisting of late vitellogenic oocytes; 41% were classified at Stage 2, with over half of observed follicles combining of early and mid-vitellogenic oocytes; 5% were classified at Stage 1, with

a majority of pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar (**Fig. 5B; SI T1; SI 2**).

In males, 45% of individuals were classified with Stage 2 testis, where spermatocytes, spermatids, and spermatozoa were present in roughly equal proportions; and 55% with Stage 3, where all stages were observed, along with mature sperm (**Fig. 5B; SI T1; SI 2**).

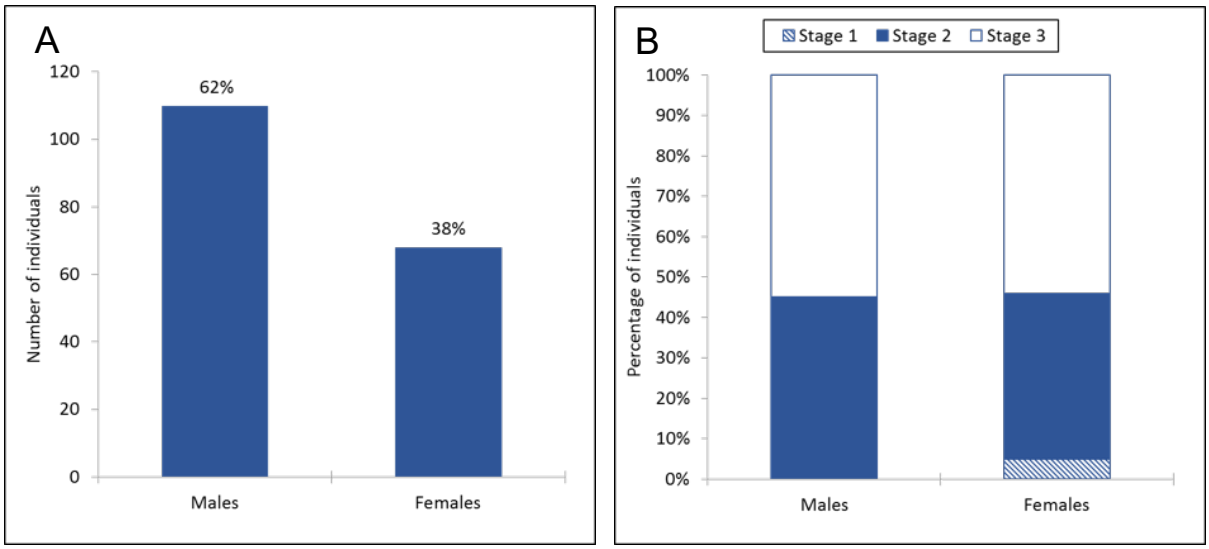

Figure 5. Sex ratios and male and female maturity index of ERE-GFP Casper zebrafish at 60dpf. Data is reported as mean ± SEM.

5.5 Discussion

Currently, there is no reliable method for distinguishing male zebrafish from females prior to the period of sexual differentiation (both morphologically and genetically), which makes the elucidation of sex determination in zebrafish problematic. In addition, in studies where sex-related sources of variation are of interest (e.g. endocrine disruption) or need to be controlled for, errors in sex identification can be a significant source of noise and bias. Thus, the generation of a reliable method for determining sex in zebrafish would prove to be an invaluable resource.

The aim of this study was to investigate whether natural endogenous GFP expression in ERE-GFP Casper zebrafish was sex related and if this could then function as an early sex marker, thus allowing the identification of putative male and females during the sex-determining period. GFP expression in fish after 30 dpf was not observed but this was most likely due to the thickness of the skin in the model, which prevented accurate quantification of GFP in the different tissue types due to fluorescence signal quenching. Levels of endogenous GFP expression were shown to differ widely during the various life stages of our zebrafish which is likely a reflection of the varying functional roles they perform during development. No definitive link was found between GFP tissue expression during early life and the phenotypic sex of an individual fish at 60 dpf, with the exception of endogenous GFP expression in the livers at 30 dpf that signalled for females.

5.5.1 GFP expression.

No endogenous GFP expression was observed in ERE-TG Casper zebrafish after 30 dpf and, as with previous work with juvenile zebrafish, is most likely due to limitations in the compound microscopy used, whereby low levels of endogenous GFP expression were not captured in later life stages. As this method is limited by absorption of excitation energy throughout the beam path resulting in out-of-focus flare, this approach would have a significantly lower threshold detection capability in later life stages, where deeper penetration into living tissue is required. Confocal or multiphoton microscopy might be considered in further studies of this nature which both provide better depth penetration. For example, confocal microscopy can reject out-of-focus background fluorescence, and construct a very precise three-dimensional (3D) image of the sample, with improved resolution horizontally as well

as vertically relative to conventional microscopy. This also applies to multiphoton microscopy, which also provides 3D optical sectioning, but without absorption, above and below the plane of focus (unlike for confocal microscopy which can exhibit photobleaching and phototoxicity). Thus, multiphoton microscopy can be used for experiments that require deep penetration into living tissue or intact animal specimens.

In this study, a small percentage of fish displayed no endogenous GFP expression throughout early development up until sexual maturity (Group A), although very low levels of endogenous GFP expression may not have been captured in later life stages due to limitations in microscopy. Interestingly, in this group the majority of fish were classified as males at 60dpf and all were found to be at an advanced stage of gonadal development, with spermatocytes, spermatids, and spermatozoa present in roughly equal proportions; along with mature sperm. The reason for a lack of observable GFP expression (from 5-30 dpf) in the only two phenotypic females found in this group is unclear but may have been linked to a slower developmental rate, as evidenced by the comparatively slow rate of gonadal development seen in both these individuals at 60 dpf - with a predominance of pre-vitellogenic follicles present in the gonads - compared with other females. As such levels of circulating endogenous oestrogens (notably E_2) may have been low for these females and insufficient during the earlier life stages to induce GFP expression, albeit this is purely speculation.

From the analysis conducted, endogenous GFP expression in the heart at 5-10 dpf as seen in Group B individuals did not appear to be related to their phenotypic sex. This might be expected as zebrafish at these life stages are considered to be bipotential hermaphrodites, and under normal conditions do not start to undergo sexual differentiation before this time period (Takahashi, 1977; Tong *et al.,* 2010). The observation of endogenous GFP expression in the heart is in agreement with previous studies by this author, and with other studies published for this and other oestrogen responsive transgenic fish lines (Gorelick *et al.,* 2011; 2014; Lee et al., 2012; Hao *et al.,* 2013). Research using ERE-GFP transgenic zebrafish, including the model in this study, has shown that the heart is particularly responsive to exogenous oestrogens, including environmental mixtures, with activation of GFP

seen from as early as 24 hpf and at exogenous exposure concentrations in the ng/L range (Gorelick *et al.,* 2011; 2014; Lee *et al.,* 2012; Green *et al.,* 2016;). This sensitivity may, in part, be a result of the high expression levels of ERs in the heart during early life stages, that may include all three isoforms (Ropero *et al.,* 2006; Pugach et al., 2016). In addition, in zebrafish, ovulated oocytes are loaded with maternal *ESR2a* mRNA, and knockdown of this gene has been shown to lead to defects in the heart (Celeghin *et al.* 2011), indicating that during early development *ESR2a* is differentially expressed in this tissue. This then, may explain the observed levels of endogenous GFP expression in the heart found in the ERE-GFP zebrafish model. Likewise, all three subtypes of ERs (*ESR1*, *ESR2a* and *ESR2b*) have also been shown to demonstrate tissue specific patterns of expression and function in early life stages; as an example, in zebrafish *ESR2a* has been reported to be differentially expressed in both the ovary and in neuromast cells, whilst *ESR1* and *ESR2b* have been shown to be selectively transcribed in developing heart valves and in the liver respectively (Menuet *et al.,* 2002; Tingaud-Sequeira *et al.,* 2004; Froehlicher *et al.,* 2009; Gorelick *et al.,* 2014). Expression of the three subtypes has also been found to partially overlap in known oestrogenic tissues such as the liver, brain, gonad, and pituitary (Bardet *et al.,* 2002; Menuet, 2002).

Endogenous GFP expression in the otic vesicle at 5-20 dpf as seen in Group C individuals was also seen to be not indicative of phenotypic gonadal sex at 60 pf. At around 10 dpf all zebrafish, regardless of their resulting gonadal sex, start to form undifferentiated ovary-like gonads (Tong *et al.,* 2010**)**. Unlike for early life stages and for adults, little information in the literature exists on the levels of ER expression during the latter stage of this period (i.e. 10-20 dpf), therefore it is hard to align these results with the current knowledge. Gonadal differentiation in zebrafish usually occurs at around 20 dpf to 40 dpf, with the juvenile ovary either degenerating and transforming into testes or continuing to develop into mature ovaries in the remaining fish (Takahashi, 1977; Uchida *et al.,* 2002). In the ERE-GFP zebrafish, a decrease in the number of individuals expressing GFP in the otic vesicle was seen between 5-20dpf, with the majority of fish (92%) found to phenotypic males at 60 dpf, and this may be linked to the transformation of the ovaries into testes, which is caused by a decrease or absence of endogenous oestrogens. However, whilst this suggests that GFP expression in the otic vesicle during this time may be related to sex, the

elucidation of phenotypic females at 60dpf in this group suggests otherwise, and this therefore cannot be used as a reliable indication of sex in the ERE-GFP zebrafish model.

In group D zebrafish, specific observation of endogenous GFP exclusively in the livers of 30 dpf female zebrafish was found which is likely a result of differences in ER signalling between the sexes. Significantly higher levels of *ESR2a* have been reported in the livers of female medaka during 30-50 dpf, with the highest expression seen at 30 dpf (Chakraborty *et al.,* 2011). In terms of gene expression, the liver is of the most sexually dimorphic organs in fish, largely because of the different metabolic needs for male and females (Zheng *et al.,* 2013). Research on the tissue distribution of the ERs in teleost fish, have shown that whilst all three subtypes are found to be induced in the liver, it is *ESR1* which is predominantly expressed (Menuet *et al.,* 2002; Griffin *et al.* 2013). Indeed, it is now widely accepted that hepatic *ESR1* is up-regulated upon exposure to oestrogens (Nelson and Habibi, 2013) and this in turn correlates very closely to the induction of vitellogenin (*VTG*; the egg yolk precursor) synthesis in the liver. Research on liver specific *VTG* genes has shown that they are normally expressed at high levels in the liver of female fish, and it is this which most likely explains the expression of endogenous GFP found in the livers of the ERE-TG female zebrafish. Conversely, baseline *VTG* levels in male fish are normally considerably lower and often non-detectable (Wang *et al.,* 2005), although it has been shown that males retain both the cellular receptors and the synthetic machinery for producing VTGs in their hepatocytes (Ankley and Johnson, 2004a).

Interestingly, not all fish identified as phenotypic females at 60 dpf in this study displayed obvious endogenous GFP expression in their livers at 30 dpf. These individuals all displayed less developed stages of ovarian differentiation and the differences in the expression of GFP during early life may therefore relate to lower levels of circulating oestrogen, but again this is speculation and would warrant investigation to see if the two variables were indeed related.

Another notable observation in this study was the apparent loss of the fluorescence in the livers of females between 30 dpf and 40 dpf. This transition may be explained by differences in levels of circulating endogenous oestrogens (notably E_2), as there

may be a spike in oestrogen production to facilitate ovary development during the critical window of sexual differentiation (which is completed by 40 dpf).

In conclusion, the results presented in this study demonstrate that endogenous GFP expression across the different body tissues during early life in the ERE-TG Casper model, and prior to gonadal differentiation is not an accurate marker for the determination of sex but could be utilised as a phenotypic marker for identifying females during the period of gonadal sexual differentiation (here at 30 dpf).

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5.9 Supplementary Information

S1. Generation of ERE-GFP-Casper (F0) line. TG1 indicates ERE-Gal4ff transgene sequence. TG2 indicates UAS-GFP transgene sequence. Expression of pigmentation genes *roy* (dark) and *nacre* (silver) are also shown. The ERE-GFP model, homozygous for both transgenes, and a Casper strain were initially crossed to give a heterozygous generation. Larvae were then screened for the homozygous ERE/Casper genotype and individuals carrying both the Casper phenotype and GFP expression were raised to sexual maturity. Fish were then screened for homozygous expression of the transgene sequences to produce the founder generation (F0) of the ERE-GFP-Casper line.

S2. Criteria for staging testes/ovary in zebrafish.

S3. ERE-GFP zebrafish ovary (AI-III) and testis (BI-II) sections at 60 dpf.

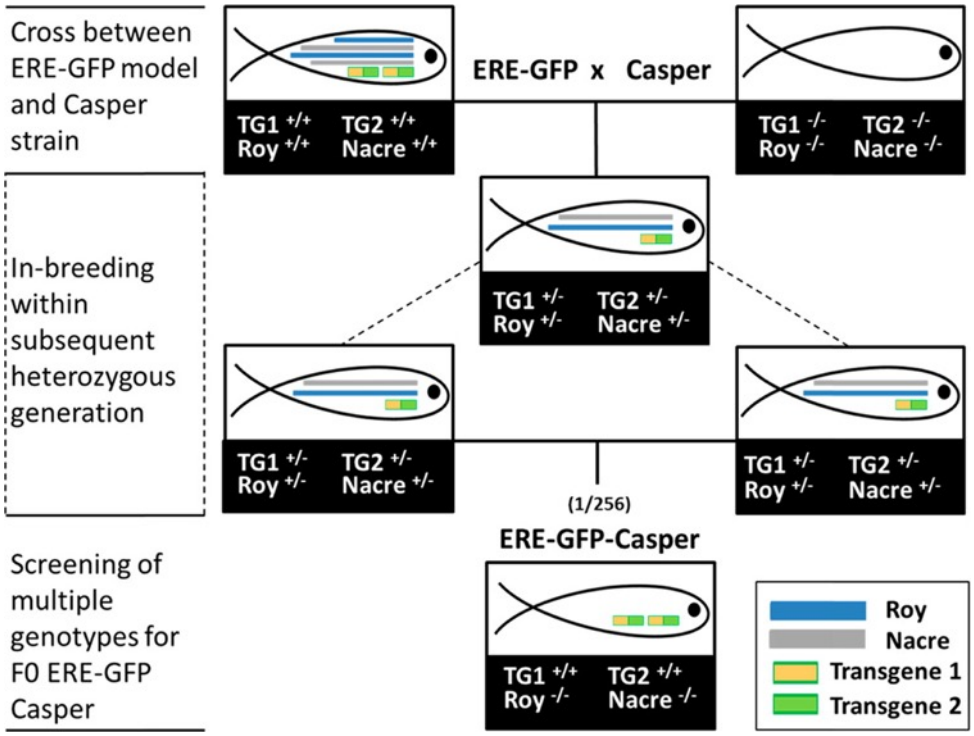
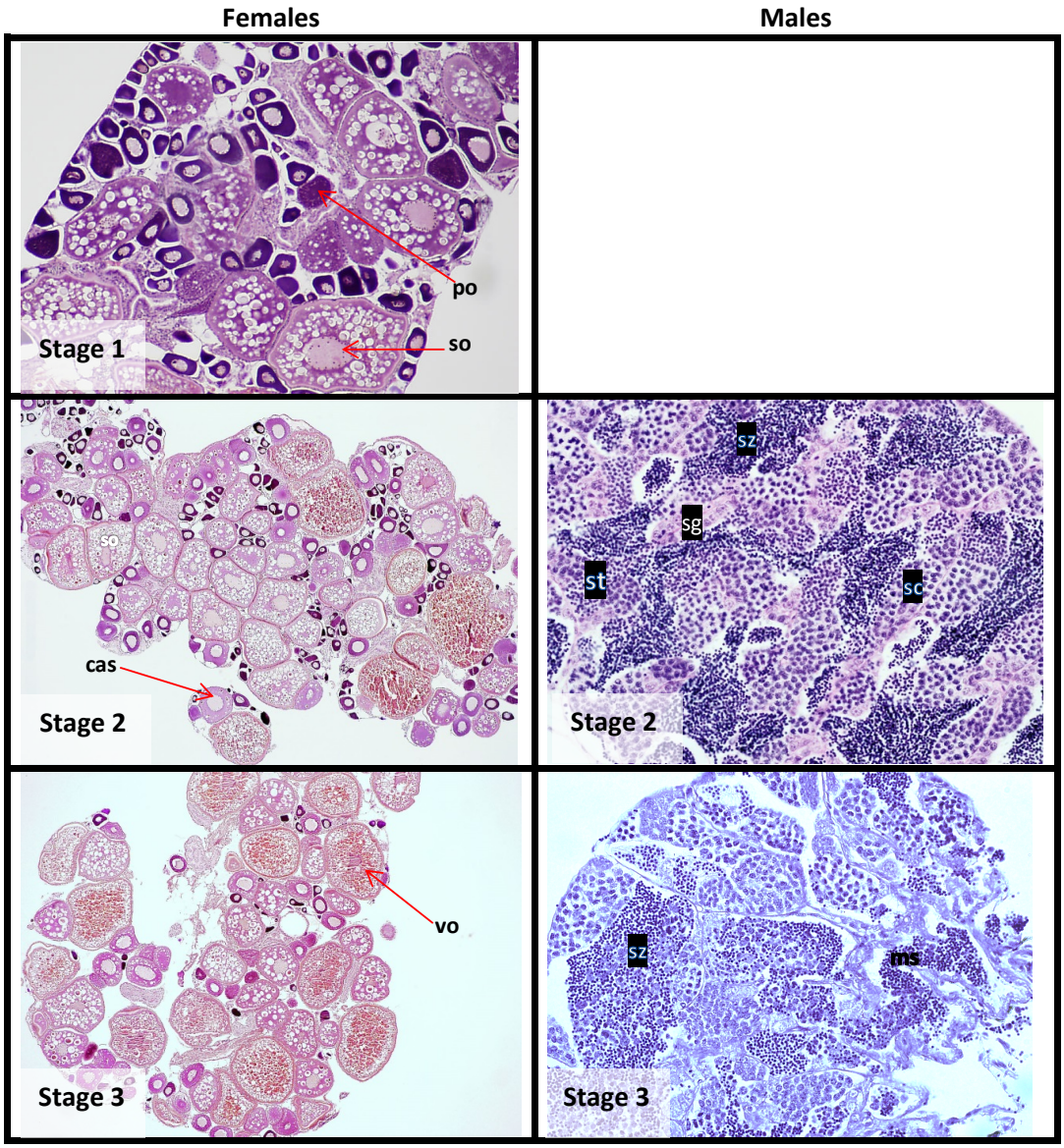

Cross between ERE-GFP model and Casper strain
In-breeding within subsequent heterozygous generation
Screening of multiple genotypes for F0 ERE-GFP Casper
ERE-GFP x Casper
TG1 +/+ Roy +/+
TG2 +/+ Nacre +/+
TG1 -/- Roy -/-
TG2 -/- Nacre -/-
TG1 +/- Roy +/-
TG2 +/- Nacre +/-
(1/256)
ERE-GFP-Casper
Roy
Nacre
Transgene 1
Transgene 2

Figure S1.

Ovary	**Stages 0-5**
	Stage 0 **–** Undeveloped - entirely immature phases (oogonia to perinucleolar oocytes); no cortical alveoli
	Stage 1 - Early spermatogenic - vast majority (e.g., > 90%) are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar.
	Stage 2 - Mid-development - at least half of observed follicles are early and mid-vitellogenic.
	Stage 3 - Late development - majority of developing follicles are late vitellogenic.
	Stage 4 - Late development/hydrated - majority of follicles are late vitellogenic and mature/spawning follicles; follicles are larger as compared to Stage 3.
	Stage 5 - Post-ovulatory - predominately spent follicles, remnants of theca externa and granulosa.
Testes	**Stages 0-4**
	Stage 0 – Undeveloped - entirely immature phases (spermatogonia to spermatids) with no spermatozoa.
	Stage 1 - Early spermatogenic - immature phases predominate, but spermatozoa may also be observed; the germinal epithelium is thinner than it is during Stage 2.
	Stage 2 - Mid-spermatogenic - spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions; the germinal epithelium is thinner than Stage 1, but thicker than Stage 3.
	Stage 3 - Late spermatogenic - all stages may be observed, however, mature sperm pre-dominate; the germinal epithelium is thinner than it is during Stage 2.
	Stage 4 – Spent - loose connective tissue with some remnant sperm.

Table S1.



Key: (ms) mature sperm, (sg) spermatogonia, (sc) spermatocytes, (sz) spermatozoa, (st) spermatids, (po) primary oocyte, (cas) cortical alveolous stage, (so) secondary oocyte, (vo) vitellogenic oocyte

Scale bars represent 100μm (female) and 20μm (male)

Figure S2

Chapter 6

General Discussion

6.1 Overview

A major route of chemical pollutants entering into the aquatic environment is via WwTW effluent discharges. One of the most prevalent groups of chemicals found in these effluents are the environmental oestrogens, and these have been increasingly associated with detrimental health effects in aquatic organisms, largely fish, as well as in humans (Iwanowicz and Blazer, 2011; Yoon *et al.,* 2014). Chemicals responsible for the oestrogenic activity in WwTW effluents include the steroidal oestrogens and manufactured compounds such as BPA and NP. However, in many cases studies have only assessed for the effects of exposure to single chemicals, and often for short time periods, hence much less is known about the health effects of complex 'real-life' mixtures and of the impacts of temporal changes in these mixes. Recently, concerns for the possible chronic health effects of oestrogenic chemicals on wild fish populations have intensified due to the uncertainty of the long-term stability of global freshwater supplies and the likelihood of lower river flows in some areas due to climate change (Johnson *et al.,* 2009**;** Keller *et al.,* 2015). These concerns are driving the need for more detailed understanding on chronic exposure effects of environmental oestrogens and also for more easily applied integrative health measures for exposures to oestrogenic chemicals and their mixtures.

Current national and international screening programs depend heavily on *in vitro* assays, consequently risk assessment of environmental oestrogens is founded on quantification of binding affinity for specific ER subtypes employing a variety of different cell lines and this does not allow for complex interactions that occur between chemicals and organisms in the environment. Transgenic fish models have been developed in recent years which have huge potential as integrative test systems, to aid further understanding of the mechanisms by which oestrogenic chemicals modify hormonal signalling pathways and how these alterations in turn may lead to chronic health effects, allowing for comprehensive effects assessment across multiple tissues *in vivo* (reviewed in Lee *et al.* 2015). Furthermore, there is the potential for the utility of these models in environmental risk assessment that is built on the adverse outcome pathway (AOP) framework (Ankley *et al.,* 2010).

In this context, the work presented in this thesis set out to investigate the ability of an ERE-GFP zebrafish model for identifying target tissues and health effects for a complex chemical mixture i.e. a WwTW effluent (including for both chronic and acute exposure periods) and for a suite of suspected oestrogens of environmental health concern. A further aim was to establish how the zebrafish model would compare, in terms of sensitivity for detecting oestrogenic responses, with vitellogenin production in fish, a well-established and sensitive biomarker for exposure to oestrogenic chemicals. Finally, the utility of the model as a potential sex marker in zebrafish was also investigated.

In doing so, this chapter presents a critical analysis of the research presented in this thesis, reviewing the key results and presenting the significance of the outcomes and their collective contribution to current understanding in this field. The challenges and limitations of the experimental work, including the use of the ERE-GFP zebrafish model for assessing the ability of oestrogenic chemicals (both individually and as part of a complex environmental mixture), are also discussed and evaluated.
A discussion on key areas of possible future research work is presented, with a foray into promising new methods that will aid scientific research in this area. Finally, a closing summary provides some overall concluding thoughts on the field as it currently stands.

Through the work in this thesis the following has been established:

(1) Temporal variability in the oestrogenic content of a WwTW effluent can be measured using the ERE-GFP zebrafish model.

In Chapters 2 and 3, a high correlation between measured steroidal oestrogen levels and GFP expression responses in the ERE-GFP zebrafish model was found throughout the different study periods, demonstrating the systems high sensitivity for assessing temporal variations in the oestrogenic content of a wastewater effluent. The effects of intermittant exposures of oestrogens (using cancerous cells), have previously been shown to be unexpectedly potent (Ponnusamy *et al.,* 2017). Thus, consideration of the variable nature of wastewater exposure when assessing the oestrogenicity of an effluent, as highlighted here, demonstrates the importance of

allowing for temporal effects when defining the influence of a wastewater effluent on a system or species. In addition, and unlike traditional chemical analysis, as a fully integrated model system the opportunity for a more effects-directed analysis approach is achievable using the ERE-GFP zebrafish, e.g. for studying the molecular mechanisms of oestrogen signal transduction.

As an aside, and within a broader context, it is worth bearing in mind that subtleties of wastewater effluent exposure are likely to work in tandem with or be exacerbated under changing environmental regimes (e.g. climate, as demonstrated in Chapters 2 and 3 in this thesis). This is especially important when considering the potential for impacts of chemical contamination on wildlife populations, particularly those deemed more at risk, i.e. small (inbred) populations or species with environmental sex determination, which have been shown to be more vulnerable to the interactive effects of climate and chemical contamination (Brown *et al.,* 2015; Luzio *et al.,* 2016).

(2) Tissue responses to oestrogenic exposures vary over time and for different developmental life stages

A wide variety of tissue types were identified as oestrogenic targets from the work in this thesis some of which have been previously reported in this model (Lee *et al.,* 2012; Green *et al.,* 2016). In Chapter 3, chronic and life-stage exposures revealed how these targets can vary over time from early life through key developmental stages up until 30dpf. In particular, results suggest that juveniles undergoing sexual differentiation may be particularly sensitive to EE_2, with a wider range of responding tissue targets compared with larval stages.

These findings add further credence to the importance of identifying specific tissue targets and of an organism's development stage with regards to potential health effects associated with oestrogen exposure (e.g. windows of sensitivity), and further demonstrates the utility of the zebrafish model for a more holistic approach to understanding effect mechanisms and for application to health effects analysis.

In addition, this study highlights an existing gap in the knowledge on the levels of ER expression during early sex differentiation (i.e. 10-20 dpf) in zebrafish and this should be investigated further.

As an aside, a novel finding from this study was the observation of GFP expression in the skeletal bone (i.e. mineralised tissue), found in 30 dpf fish exposed to $EE_{2,}$ and previously unreported in other transgenic oestrogen responsive fish models. This demonstrates how the ability for the visualisation of target tissues in the ERE-GFP model can be employed to clarify whether an inferred response to an oestrogenic chemical is mediated via the ERs, through the activation of the oestrogen signalling cascade in these cells. Furthermore, it also highlights the importance of transgenic fish models in ecotoxicology studies that do not have markers reliant on individual tissues, such as a vtg or cyp19a1b promotor that only express in the liver and brain respectively (Bogers *et al.* 2006**,** Petersen *et al.* 2013). However, although extremely useful in identifying specific tissue targets and determining signalling pathways, it must be stressed that a fluorescent response in the ERE-GFP zebrafish is not necessarily indicative of a health effect in these tissues, thus a need for caution in interpreting results should always be applied.

(3) The ERE-GFP zebrafish model is a viable system to detect for the presence of environmental oestrogens and health effects in a complex mixture (e.g. a WwTW effluent).

Although several transgenic fish lines have been developed for use in ecotoxicological testing, most studies have been limited to single chemical exposures (focusing on the generation of the model), with few having advanced to the stage where they are routinely used in empirical research (see review Lee *et al.*, 2015). This is the first study that has successfully demonstrated the suitability of an integrated approach coupling effect-directed analysis with an ERE-GFP transgenic fish model with which to characterise oestrogenic chemical contamination and health risks associated with a wastewater effluent. Furthermore, this model offers the potential to facilitate the simultaneous understanding of chemical mechanisms of action and adverse outcome analyses from the molecular to the organismal level, thus proving its viability as a tractable system which can be useful to the fields of ecotoxicology and environmental risk assessment (ERA).

Screening chemicals by using a conventional biomarker approach such as VTG induction can be problematic as it involves extensive animal use and yields little information on mechanistic toxicity (Ankley *et al.,* 2010; Kramer *et al.,* 2011; Volz *et al.,* 2011). Whilst the results presented here suggest that GFP induction is less sensitive than VTG induction, in terms of the magnitude of response, the positive correlation between the two variables are indicative of the considerable potential of the ERE-GFP zebrafish as a responsive biomarker system for the detection of oestrogens particularly in complex real-world chemical mixtures.

(4) Molecular initiating events (MIEs), and/or cellular, organ and organism level key events (KEs) within an AOP framework can be evidenced or inferred using the ERE-GFP zebrafish model.

Work in Chapters 2, 3 and 4 using both individual environmental oestrogens and complex mixtures (i.e. a WwTW effluent) have shown the potential of the ERE-GFP zebrafish model for defining AOPs for this group of chemicals **(Fig. 1).**

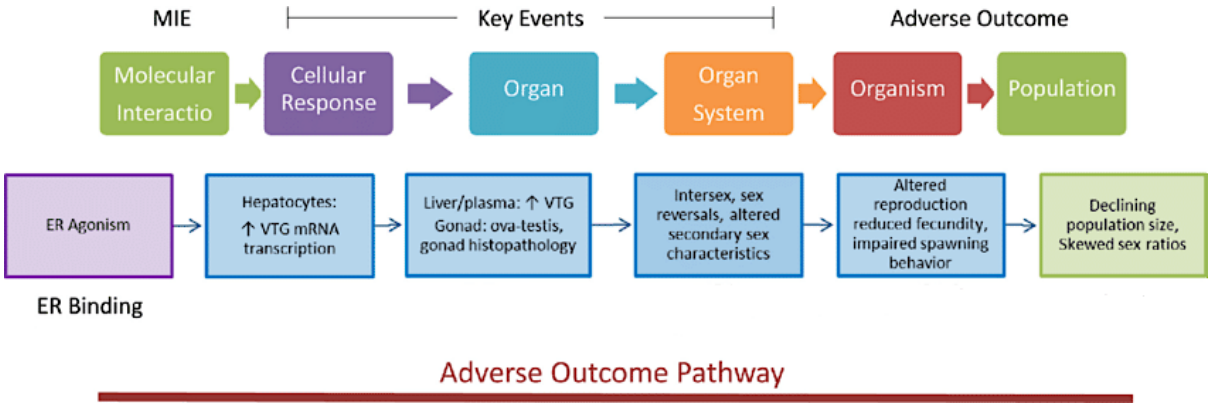


Fig. 1. Oestrogen receptor (ER) binding and activation (i.e. MIE) can be linked to several key events leading to an adverse outcome in the ERE-GFP zebrafish models used in this thesis. (Source: US EPA, 2018)

This is important as if significant MIEs or KEs are revealed and can subsequently be strongly linked to certain AOs via the use of the ERE-GFP zebrafish model, existing alternative tests that can inform on the capability of the identified chemical(s) to cause the identified events could then be used as a replacement for whole animal

testing where available. Conversely, this may require more animal testing initially to help facilitate any knowledge gaps identified during AOP development but will ultimately allow for an improved mechanistic understanding of linkages across biological scales with a resultant reduction and replacement of animals in the longer term.

In particular, early life stage development AOPs formed around endpoints that can be measured in the zebrafish embryo test could be used to support chemical screening and prioritisation guidelines (Volz *et al.,* 2011). However, it is worth noting that for complex mixtures such as wastewater effluents, longer-term exposure times (i.e. embryo to adult toxicity tests) may also be warranted in order to determine any potential AOs.

(5) Biological responses to both the oestrogenic effluent and the synthetic oestrogen EE_2 can differ between the sexes

In Chapter 3, chronic exposure to both 100% effluent and EE_2 from early fertilisation to sexual maturation (0-90dpf) was shown to delay the maturation of testis in males, with the opposite effect observed in females (i.e. accelerated gonadal development). In addition, growth effects were more pronounced in males than in females, with significant smaller sized fish seen for exposures to EE_2 and 100% effluent. These findings were consistent for all developmental exposure periods and are important as they demonstrate how oestrogens can elicit distinct effects dependent on sex. From an AOP perspective, impaired development or growth at the organism level can have subsequent implications for population level effects (Johnson and Hixon, 2011). Thus, studies such as these provide valuable information regarding the potential health effects of environmental oestrogens on sexual differentiation in fish caused by agonism or antagonism at the ER level.

As an aside, given the case that both juvenile and adult female zebrafish have varying but significant levels of natural circulating oestrogen, it is suggested that exposure studies with the ERE-GFP zebrafish during these life stages, is perhaps only practical (and more applicable) in males. However, due to the lack of availability of a genetic sex marker, which has yet to be forthcoming for zebrafish, distinguishing

between males and females before sexual maturation is not yet possible without destructive sampling.

(6) Endogenous GFP expression in the ERE-GFP zebrafish model does not appear to be an accurate marker for sex determination, at least in terms of early life stages.

In Chapter 5, gonadal sex in the ERE-GFP Casper zebrafish model was only linked to the pattern (intensity) of GFP expression in the livers of fish at 30dpf, which correlated with phenotypic females. This life period coincides with the timing of sexual differentiation of the gonad in zebrafish. Whilst these results show that endogenous GFP expression cannot be used to distinguish gonadal sex in this transgenic zebrafish line prior to gonadal differentiation using fluorescence microscopy, it could however be applied as a phenotypic marker for non-destructively identifying females at 30dpf. This could benefit studies such as those carried out in this thesis where sexual difference needs to be accounted for (e.g. endocrine disruption) or needs to be controlled for, i.e. errors in sex identification can be a significant source of noise and bias. However, further work with this model is warranted during the period of sexual differentiation, providing limitations in microscopy are addressed, allowing accurate quantification of GFP in these life stages and this is discussed later in this chapter.

6.2 Challenges and limitations of the work undertaken

This thesis has presented a range of novel findings as stated above, which make a valuable contribution to the current understanding in this field. However, that is not to say that there have not been any inherent limitations/challenges to the work undertaken, particularly in relation to the ERE-GFP zebrafish model, some of which can/have already been overcome with further investigation.

(1) Whilst our ERE-GFP model can offer visual quantification of tissue targets of oestrogenic chemicals through image analysis microscopy, this is laborious and time consuming (i.e. orientation of the larvae). Since the start of this thesis however, a semi-automated systems microarray has been developed with our ERE-GFP-Casper model which would help to alleviate this problem, although the detection monitors in

the automated system are not as sensitive as required to detect the lowest level GFP expression (Green *et al.,* 2016).

(2) A major limitation of the original ERE-GFP zebrafish line was the inability to observe GFP expression in live fish post 5dpf using fluorescent microscopy, imposing limits of possible experimental work. Whilst the generation of the pigment free ERE-GFP-Casper has helped to alleviate this problem, the thickness of the body wall (post 30dpf) diminishes visualisation of fluorescence signals for deeper seated tissues in the body cavity for effects analysis. As such the use of confocal or multiphoton microscopy, which may provide better depth penetration could be employed to investigate if this issue can be circumvented, thus allowing the utilisation of the ERE-GFP-Casper for health effects analysis in adult life stages. However, these methods are more technically challenging as standard fluorescence microscopy. A similar zebrafish line named “Crystal” has been developed that also includes silenced pigmentation in the eyes, further enhancing imaging of the brain and may provide improved translucency at later developmental life stages than the Casper phenotype (Antinucci and Hindges, 2016).

(3) Although the zebrafish models presented in this thesis have been shown to be comparable (in terms of sensitivity) to currently available *in vivo* and *in vitro* oestrogen responsive systems (Soto *et al.,* 2006; Dobbins *et al.,* 2008; Dreier *et al.,* 2015) as they only express one type of transgenic ERE and surrounding sequence they will only be effective for identifying chemicals which exert oestrogenic effects through the classical (genomic) pathway. Thus, they will not pick up interactions of ERs that work through other signalling pathways (e.g. Sp1 or AP1 sites) or non-genomic and ligand independent pathways, or indeed anti-androgens which can induce similar phenotypes to those observed through exposure to oestrogens, e.g. de-masculinisation of male fish. This can be problematic when trying to assess potential health effects in wastewater effluents which contain complex mixtures of chemicals.

(4) To date there has been little effort to directly compare responses in transgenic fish with wild type lines. This is important in the context of establishing the potential health effects of oestrogenic chemicals on fish populations more generally, particularly with regards to reproductive endpoints such as intersex in fish.

For example, it has recently been suggested that domesticated strains of zebrafish have lost the natural sex determinant retained by their wild type cousins, postulated to be a female WZ male ZZ sex determination mechanism (Wilson *et al.,* 2014), instead possessing a complicated polygenic sex determination system. Therefore, it is critical that future studies in this field should also strive to include work with natural strains containing the wild sex determinants. This would provide a better understanding of the similarities and differences in life and reproductive strategies between a transgenic model and the wild fish species of interest. Likewise, laboratory zebrafish strains are generally more inbred and therefore less genetically diverse than their wild cousins, thus their value as a model in predicting the impacts of chemical exposure on native wild populations is questionable (Coe *et al.,* 2008a; Lawrence *et al.,* 2008; Whiteley *et al.,* 2011). Indeed, previous studies do suggest that susceptibility to chemical exposure is influenced greatly by genetic factors. For example, responses to chemical exposures have been shown to change as a result of reduced genetic diversity, e.g. fewer reproductive females compared to outbred fish (Brown *et al.*, 2011), and lower embryo viability (Bickley *et al.*, 2013). It is also worth noting that the genetic origin of a laboratory strain could affect the results of chemical exposure studies, i.e. whether the ancestral population had previously been exposed to pollution.

6.3 Future perspectives

The research presented in this thesis provides groundwork establishing the potential for health effects of a complex chemical mixture i.e. an oestrogenic WwTW effluent (including for both chronic and acute exposure periods) and how these responses can alter over time and between different developmental stages. The following section now identifies key areas of future research with regards to transgenic fish models and establishing the potential health effects of exogenous oestrogens.

1. Identifying the molecular mechanisms of estrogenic chemicals. As a fully integrated model system, the ERE-GFP zebrafish offers the potential for studying the molecular mechanisms of oestrogen signal transduction. Characterising how oestrogenic chemicals may affect different signalling pathways *in vivo* is a difficult task, further confounded by the fact that there may be cross talk between different

cellular signalling pathways. However, this is an important area of future research to fully understand the possible health effects of these compounds. The use of morpholinos for antagonising specific ER subtypes, or CRISPR-Cas or TALENS (used to silence ER subtype expression) in the responsive target tissues identified in this thesis in the ERE-GFP model could aid in the clarification of the exact mechanisms of action for different environmental oestrogens and is therefore highly recommended.

2. Developing the use of the ERE-GFP zebrafish model for environmental risk assessments. The use of ERE-GFP-Casper embryos in high-throughput screens has already been shown to be possible, under a variety of exposures, using an array scanner and image analysis (Green *et al.,* 2016), however in order for the system to be recognised as part of general regulatory guideline chemical tests, direct comparison of biological responses in the ERE-GFP zebrafish model with wildtype lines should be carried out to establish how the nature of the promoter used in the model differs from natural promoters. This is crucial for establishing confidence that transgenic fish are also representative of the current lines of fish typically used in chemical testing. In particular, the use of early life stage embryos instead of adult fish will result in a potential reduction of intact animals used in animal testing, in line with the values of the 3Rs of reduction, refinement and replacement. This is particularly pertinent for effluent testing, where the number of vertebrates used is believed to be much larger than that for individual chemical testing (Norberg-King *et al.,* 2018). The work in this thesis has also shown great potential for defining AOPs for chemicals with the aid of transgenic zebrafish models and this should be explored further.

3. Whole-genome sequencing in established target tissues. Target tissues should be sequenced to establish oestrogen molecular signalling pathways, for example, using second generation sequencing techniques to inform on potential biological effect outcome(s). Although this thesis has identified oestrogen responses in many tissues, future studies should focus on the responses in the heart, skeletal muscle and forebrain (in larvae) where effects of environmental oestrogens have yet to receive major attention, but for where adverse health exposure effects have been reported (e.g. BPA and coronary impacts in humans, [Gao and Wang, 2014]). This

may prove challenging in embryonic/larval tissues but could be achieved with the use of techniques such as fluorescent activated cell sorting (FACS), which would allow isolation of GFP expressing cells, although this would depend on the tissue of interest and the level of RNA/DNA required. Molecular sequencing (i.e. ATAC-seq) could then be used to determine gene transcription. Further bio-informatic analyses would then be required to construct potential functional effect pathways in the target tissues.

4. Combining different reporter systems in transgenic fish models. Integrating different reporter systems in the same transgenic model, each with different fluorescent markers, could potentially allow for numerous toxicological responses (coupled to multiple signalling pathways) in the same organism, e.g. ERE and *cyp19* gene promoters. The *cyp19* gene encodes P450 aromatase, the enzyme catalysing the conversion of oestrogens from androgens and is highly expressed in the brain and ovary of zebrafish (Tong *et al.,* 2001). Hence, combining both promoters would provide for more holistic information on the roles and effects of both natural oestrogens and of oestrogenic chemicals in fish.

5. Combining multiple stressors of effects. Future studies of the potential health effects of exogenous oestrogens should aim to encompass multiple stressors (e.g. environmental factors such as temperature, PH) in combination with multiple endpoints of species in an effort to try and replicate a more realistic scenario to aid with the wider management of environmental chemical pollution. Furthermore, this approach could be incorporated into the AOP framework for oestrogen receptor activation, for which there exists comparatively robust data depicting the underlying targets and mechanisms of endocrine disruption for several chemicals, although not all are fully understood (Ankley *et al.,* 2010). Incorporating other stressors into AOPs, for example environmental exposure components such as climate (Hooper *et al.,* 2013; Moe *et al.,* 2013) could be used to help predict both how and when chemical and environmental stressor interactions might occur. This could then be taken forward as a means for identifying species, populations, and geographical regions that may warrant further study and protection, as opposed to relying on sets of disparate empirical data with which to guide regulatory decision-making and testing.

6.4 Final thoughts and comments

As the production, use and disposal of chemicals in todays' modern society continues to grow, it is worth reflecting that perhaps future research would be best spent focusing on the design of "greener" chemicals without biological or hormonal activity similar to those of natural oestrogens. This would necessitate an integrative multi-disciplinary approach in cooperation with industrial partners, where efficient screening methods are employed prior to mass manufacture. This in turn will help aid the process of protecting the environment in a manageable way.

Whilst there is a considerable research effort into the adverse health effects of oestrogenic chemicals in freshwater teleost fish species, there is potential that this could lead to an inherent bias when establishing the causes of feminisation/intersex in fish in particular. Exposures to anti-androgenic chemicals which are also found in wastewater effluents, can result in sex reversal in male fish and amphibians, but this has so far received less attention (Bayley *et al.,* 2002; Orton *et al.,* 2018). Anti-androgenic chemicals act by inhibiting the androgen receptor, resulting in molecular events that include preventing the production of testosterone (Kortenkamp and Faust, 2010). Research in this field would therefore benefit from the generation of an androgenic transgenic zebrafish model in order to establish if chemicals can exert effects via oestrogenic and/or androgenic pathways.

Effect analysis of wastewater effluents should strive to be applicable for a wide range of aquatic environments (e.g. freshwater vs marine, temperate vs tropical) and include considerations for interspecific differences. For example, despite evidence of the variability in sensitivity of different fish species to a wide variety of chemicals (Van den Belt *et al.* 2003; DeForest *et al.* 2012; Lange *et al.* 2012), most species used in ecotoxicological studies are selected for practical reasons, such as small size, high fecundity, short generation time, cost, etc. With over 33,000 described species, fish exhibit greater phylogenetic diversity than any other group of vertebrates (Fishbase, 2018), thus it should be asked as to how widely the results from any study can be applied. Consequently, future work needs to be developed for other species that better represent the phylogenetic diversity of fish. Oestrogen signalling has also been reported in invertebrates (Keay and Thornton, 2009), with growing evidence also which suggests that oestrogenic effects can be caused by

non-ER-mediated pathways (Janer and Porte, 2007; Oehlmann *et al.,* 2007). Thus, studies are required to fully understand toxicity mechanisms across species.

Recently, projections based on a growing worldwide population and climate change, alluded to a potential two-fold rise in oestrogenic contamination of surface waters by 2050 (Green *et al.* 2013), thus presenting a greater risk for aquatic organisms. The potential for clearer, more comprehensive information can be achieved across ecotoxicology exposures by employing a more holistic approach, rather than focusing on a single process. It is vital to progress beyond the piecemeal, one chemical at a time, one dose approach currently used in many studies and in ERA.

Understanding the effects of chemical mixtures to which aquatic wildlife and humans are exposed is increasingly important and needs to take into account the characteristics of the endocrine system that are being disrupted, including tissue specificity and sensitive windows of exposure across the lifespan. This alternative, effect-based approach is increasingly important in regulatory assessment of wastewater effluents and has gained growing international interest under initiatives such as the European Union Water Framework Directive as a way of furthering the risk assessment of chemical mixtures present in surface waters (Brack *et al.,* 2017).

Ultimately, the results of this thesis, supported by future work outlined in this discussion, further highlights how the development and application of transgenic fish models can offer huge potential for more integrative health effects assessments, allowing for an improved understanding of the risks posed by oestrogenic chemicals, and for tailoring future approaches to ERA strategies, specifically in respect of AOP frameworks.

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